MITOCHONDRIAL RNA SYNTHESIS AND RIBONUCLEOTIDE INCORPORATION STUDIES IN *Euglena gracilis*

by

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This dissertation is dedicated to my wife, Carolyn M. Brown, my parents, Mr. and Mrs. Marshall V. Brown, and my children, George E. Brown and Erika N. Brown, for their endless encouragement and personal sacrifices during the time I spent in graduate school.

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KEY TO ABBREVIATIONS

A_{260}	absorbancy at 260 nm
A_{280}	absorbancy at 280 nm
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BGP- 1	the first peak of tridium labeled material eluted from Biogel P-4 column
BGP- 2	the second peak of tridium labeled material eluted from Biogel P-4 column
BSA	bovine serum albumin
CAP	catabolite gene-activator protein
CDP-diglyceride	cytidine diphosphate diglyceride
CMP	cytidine monophosphate
CpC	cytidylyl (3' 5') cytidine
cpm	counts per minute
CTP	cytidine triphosphate
dCTP	deoxycytidine triphosphate
cyclic AMP	adenosine 3' : 5'-cyclic monophosphoric acid
DEAE	diethylaminoethane
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease (EC 3.1.4.5)
dpm	disintegrations per minute
DTT	dithiothreitol

EDTA	ethylenediamine tetraacetate
g	gravity
GMP	guanosine monophosphate
GTP	guanosine triphosphate
hr	hour
^{3}H	radioactively labeled with tridium
I	Iodine
NTPs	ATP, CTP, GTP, UTP (minus the labeled substrate)
poly d(AT)	alternating copolymers of deoxyadenylic and deoxythymidylic acid
poly (C)	poly cytidylic acid
POP	2, 5 - diphenyloxazole
POPOP	1, 4 - di-[2-(5-phenyloxazolyl)]-benzene
RNA	ribonucleic acid
Hn RNA	heterogeneous nuclear ribonucleic acid
m RNA	messenger ribonucleic acid
r RNA	ribosomal ribonucleic acid
t RNA	transfer ribonucleic acid
RNase	ribonuclease (EC 2.7.7.16)
S	sedimentation coefficient
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
SVP	snake venom phosphodiesterase
TCA	trichloroacetic acid
tris	tris (hydroxymethyl amino) methane
UMP	uridine monophosphate
UTP	uridine triphosphate
UV	ultra violet

Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

MITOCHONDRIAL RNA SYNTHESIS AND RIBONUCLEOTIDE INCORPORATION STUDIES IN *Euglena gracilis*

By

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Chairman: James F. Preston, Ph.D
Major Department: Microbiology

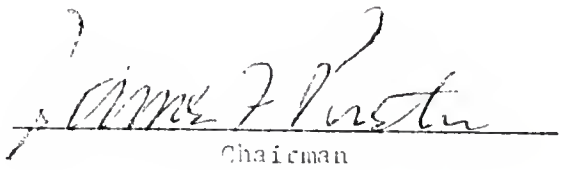
Two experimental approaches were utilized to study the *in vitro* mitochondrial RNA synthesis in *Euglena gracilis*. First the RNA synthesis activity was examined in isolated and highly purified mitochondria of *Euglena gracilis* strain z streptomycin bleached aplastic mutants. The mitochondria obtained after isopynic centrifugation in sodium diatrizoate gradients yield mitochondrial DNA (ρ=1.691 g/cm^3) and little nuclear DNA (ρ=1.707 g/cm^3). The incorporation of label from the ^{3}H-ribonucleoside triphosphates into acid insoluble products showed no dependence upon the presence of the other ribonucleotides or that of added DNA. The preferred substrate was ^{3}H-CTP from which label was incorporated into acid insoluble products at a rate 100 times greater than the other labeled ribonucleotides. This activity incorporates CMP from CTP into acid insoluble products

which contain phosphodiester bonds by a pyrophosphorolysis reaction. The activity was inhibited by pyrophosphate and actinomycin D at 100 mg/ml but not by inorganic phosphate, α-amanitin, rifampicin or low concentrations of actinomycin D (50 mg/ml). The products were sensitive to snake venom phosphodiesterase and alkaline hydrolysis but not pancreatic ribonuclease. The products were isolated by phenol extraction followed by ethanol precipitation of the aqueous phase. Two classes of molecules with mean molecular weights of 800 and 680 respectively were isolated. They were found to have absorbancy spectra identical to that of C_pC and were resistant to pancreatic ribonuclease digestion. However, snake venom phosphodiesterase cleaved the products into 5'-CMP, the expected cleavage product of C_pC or poly C.

Mitochondria, purified by either isopycnic centrifugation or by treatment with pancreatic deoxyribonuclease, contained an associated activity which incorporated label from ^{3}H-UTP into acid insoluble products which were sensitive to pancreatic ribonuclease digestion. This activity was DNA dependent in the DNase mitochondria which was in contrast to that observed for sodium diatrizoate purified mitochondria.

The second approach was to study the activity of solubilized and partially purified mitochondrial RNA polymerase. An enzyme purification procedure is described utilizing a solution of Triton X-100 detergent and KCl, centrifugation, ammonium sulfate precipitation, and DEAE-Sephadex A-25 chromotography that resulted in a partially purified enzyme with a specific activity of 0.3 nmoles UTP/mg/10 min at 37°. The incorporation of UMP into acid insoluble material required DNA the four ribonucleoside triphosphates: GTP, CTP, UTP, ATP, and metal, either Mn^{++} or Mg^{++}. The product of the reaction was sensitive to pancreatic ribonuclease digestion. The mito-

chondrial enzyme is distinctly different from the nuclear RNA polymerase II with respect to chromatographic behavior and antibiotic sensitivity. It eluted from the DEAE-Sephadex column in a single peak between 0.32 M and 0.37 M ammonium sulfate in TGMED buffer, was stimulated by low concentration of the metals Mn^{++} and was insensitive to α-amanitin and rifampicin.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INTRODUCTION

Informational macromolecules are those through which genetic information actually flows. Genetic information may be defined as that primarily required to assemble a protein needed to perpetuate biological order, but the definition applies also to the information for ribonucleic acids (RNAs) which are not translated into protein. Deoxyribonucleic acids (DNA) functions as the repository for information or coding sequences ultimately destined to appear either in RNA or protein, and in noncoding sequences which can act as signals for such things as initiation and termination of the biosynthesis of a RNA chain. Within the cell the informational sequences are transmitted on which specific proteins are synthesized.

The DNA dependent RNA polymerases (E. C. 2.7.7.6.) are the key enzymes implicated in the first step of genetic information flow from DNA to protein. The term DNA dependent RNA polymerases is used to designate enzymic activities which, using DNA templates, catalyze the sequential assembly of the four ribonucleoside triphosphates into RNA molecules. The synthetic reaction requires the presence of a DNA or a polydeosyribonucleotide template and a divalent metal ion. The main features of the transcription reaction consist of the enzyme locating and binding to specific sites on the DNA, initiating a RNA chain de novo, extending the chain by moving along the DNA using only one strand as a template to direct the polymerization of the four ribonucleoside triphosphates into

a complementary RNA molecule, and finally, in some cases, termination of the chain with the release of the enzyme from the DNA template. RNA polymerases are responsible for the synthesis of ribosomal RNA's, transfer RNA's and messenger RNA's, all of which are needed for translation of the mRNA template into proteins.

Substantial data have been accumulated on the regulation of RNA polymerases in prokaryotes, where a single enzyme is responsible for the synthesis of all types of cellular RNA, but comparatively little is known in the case of eukaryotes, where the situation is more complex. Within the last few years, it has been demonstrated that eukaryotic cells contain several types of RNA polymerases, which differ in their localizations, structures, and functions.

Nuclei contain several different RNA polymerases. Nuclear RNA polymerase I is localized in the nucleolus (1). It is primarily concerned with the transcription of genes specifying the larger (45s) ribosomal precursor RNA. Nuclear RNA polymerase II is localized in the nucleoplasm and presumably transcribes messenger RNA. It is inhibited by α-amanitin (2,3) an octapeptide produced by several species of the genus Amanita (4). In several organisms a third (5,6) or even a fourth (7,8) nuclear RNA polymerase activity was found. A separate RNA polymerase III, which is distinct from forms I and II, has been shown to transcribe (4s) t-RNA and 5s RNA (9,10). In mammals this enzyme is slightly sensitive to α-amanitin, being 1,000 fold more resisistant than RNA polymerase II.

RNA polymerase activity has also been found localized in mitochondria and chloroplasts. The mitochondrial enzymes exhibit properties

which clearly distinguish them from the nuclear RNA polymerases. The mitochondrial enzyme is insensitive to α-amanitin and although some functional differences have been described in mitochondrial RNA polymerase, there is general agreement that the molecular weight of the enzyme, consisting of a single polypeptide chain, is about 60,000 daltons (11-15).

Control of RNA synthesis for regulating cell growth, development, and differentiation

If considerations were based purely on energy requirements, it would be reasonable to exclude DNA replication and mRNA translation as primary levels where gene expression is controlled. Several reasons may be given for excluding changes in the kinds or numbers of genes and post-transcriptional modification as steps at which regulation generally takes place. Nuclei of cells in different stages of development in the same individual appear to be genetically identical and changes in the number of gene copies from which mRNA is transcribed have not been observed. Likewise, there is no good evidence that changes in the kinds of proteins synthesized in development can be attributed to controls at the translational level and in addition eukaryotic cells have no mechanism for excluding the translation of messages characteristic of other cell types (16). It seems that translational controls are used to make quantitative adjustments to a pattern of protein synthesis determined primarily by the synthesis of new messages.

It can be concluded by elimination that transcription must be the level at which gene expression is primarily controlled. In bacterial systems, there are two well-characterized examples of programmed trans-

scription: bacteriophage development and bacterial sporulation. These processes are similar in some ways to differentiation. Therefore, understanding the control of transcription in these processes may provide some clues to the regulation of transcription in eukaryotic systems. Studies of transcriptive regulation of phage λ show how the expression of specific genes is regulated by other gene products (17). In both T_4 bacteriophage development and Bacillus subtilis sporulation, significant changes that are coordinated with development occur in the RNA polymerase molecule and associated proteins (18,19,20). In B. subtilis there is evidence suggesting that these changes may be related to the onset of sporulation.

The primary need for regulating transcription is that, by turning it on and off, protein synthesis is controlled at the origin. In both prokaryotic and eukaryotic organisms regulation must enable cells to cope with fluctuation in their immediate environment. In the prokaryote most of the genes come into operation sooner or later during each cell cycle, but this is not true of many genes in the higher eukaryote where differentiation has resulted in a strategy of regulation unlike that in the prokaryote. By contrast the lower unicellular eukaryote has a regulatory resemblance to the prokaryote. Evidence that gene transcription is controlled in eukaryotic development is provided by the Balbiani rings of polytene chromosomes. The ring is characteristically expanded and active in RNA synthesis or unpuffed and inactive in transcription at different stages of development (19). Also, agents such as actinomycin which inhibit RNA synthesis but which have little immediate effect on translation arrest eukaryotic development within a short time. These effects suggest an essential role for the regulation of transcription in development.

Regulation of transcription by alteration in molecular properties of RNA polymerase

The regulation of transcription may be affected by control of any of the components involved in the RNA synthesis: the template, the polymerase, associated factors and other environmental conditions. However, the multiplicity and different intranuclear localization of DNA-dependent RNA polymerases have suggested that gene expression in eukaryotic cells is regulated, in part, by distinct RNA polymerases with different template specificities. The quantity and quality of RNA synthesis can depend either on the concentration of RNA polymerase or on the level of activity of the enzyme. In growing E. coli cells, there is a pool of excess RNA polymerase (21) although the polymerase subunits seem to be synthesized at the same rate as other cellular proteins (22). Therefore, the regulation of growth depends upon modulation of the RNA polymerase activity rather than upon the concentration of the enzyme. Although the rRNA genes account for only about 0.4% of the genome (23), rRNA accounts for 40% of the total RNA synthesized in growing cells and is therefore preferentially synthesized (24).

On the other hand, bacteriophage infection results in the inhibition of host cell transcription presumably by the destruction or alteration of the host RNA polymerase (25,26). Similar effects on host RNA synthesis have been found in viral infections in mammalian cells (27,28). This may be analogous to terminal differentiation where the loss of nuclear function is involved (29)

Regulation of transcription and control of organelle development

An understanding of the mechanisms by which a eukaryotic cell controls organelle development should give valuable insight into many

similar cellular control processes such as those involved in differentiation, morphogenesis and tumor production. Since RNA polymerase has been implicated in controlling development in prokaryotic systems (18,19,25, 30,31) it could be a major controlling factor in Euglena gracilis organelle development. Therefore, the research in this laboratory focuses upon the RNA polymerases found in Euglena, and upon their role in organelle development. Euglena is an excellent organism to study transcription in organelles since it contains both chloroplasts and mitochondria. The organism is a unicellular flagellate with 10 to 12 chloroplast per cell, numerous mitochondria, and a polyploid nucleus. Euglena can be grown phototrophically, heterotrophically or mixotrophically. These growth conditions will alter the development of the organelles and therefore offer a useful system for studying organelle development. Phototrophic conditions are restrictive for photosynthetic function, but the other two growth conditions are permissive. In cells grown phototrophically, mitochondrial development is repressed, while the chloroplasts function to provide the bulk of the cell's energy. Cells grown mixotrophically derive energy from functional chloroplasts and mitochondria. However, wild type cells can be grown in the dark as etiolated heterotrophs where the proplastids, which have stopped forming chlorophyll and chloroplast membrane, exist as undeveloped proplastids which are capable of differentiating into functional mature chloroplasts upon exposure to light. The heterotrophic dark grown cells primarily use mitochondrial oxidative phosphorylation as a source of energy. It has been shown (32) that cells grown heterotrophically have large active mitochondria, and that exposure to light, which starts plastid develop-

ment, results in a decrease in mitochondrial density and function (33). Mutants that completely lack plastids (aplastidic) have been produced by treatment with streptomycin, heat, or ultraviolet light (34,35). These permanently bleached cells are perfectly viable as long as they are grown on a carbon source which can be respired. This makes it feasible to distinguish processes controlling mitochondrial development from those controlling chloroplast development.

This dissertation concerns itself with studies of mitochondrial RNA synthesis in Euglena gracilis and is directed towards elucidating the components of the transcriptional process, in particular, characterizing the mitochondrial DNA dependent RNA polymerase with a view toward understanding the control of transcription. The RNA synthesizing activity of isolated mitochondria has been studied and the DNA dependent RNA polymerase has been solubilized and partially purified. The experimental approach in this study was to develop a method for preparing highly purified mitochondria, to study the incorporation of labeled ribonucleoside triphosphate precursors into RNA by isolated mitochondria and to identify the labeled products. The mitochondrial DNA-directed RNA polymerase was partially purified by ion exchange chromatography. The enzyme activity was characterized to determine its requirements for product synthesis and to compare it to nuclear activities with respect to these requirements.

LITERATURE REVEIW

Guides to the Literature

In recent years, several review articles or symposia on RNA polymerases from prokaryotes (20,36-46) and eukaryotes (47-52), on the regulatory elements (53,54) and on transcription (55-59), have appeared. There are several introductory texts (60-62) and collected papers (63-66) which may be consulted for a more historical background discussion.

General Mechanisms of RNA Synthesis

The synthesis of RNA from DNA is mediated by a DNA dependent RNA polymerase that uses ribonucleoside triphosphates as precursors. In general the reaction involves binding of enzyme to DNA template and the asymmetric transcription of the DNA. RNA synthesis appears to proceed in the following steps: template binding, chain initiation, chain elongation and termination. The RNA polymerase binds to specific initiation sites (promoter sites) on the DNA in a specific reaction in which the strands of the DNA are opened over a short local region. Chain initiation involves the binding of two ribonucleotide triphosphates to the RNA polymerase followed by elimination of inorganic pyrophosphate to form a dinucleotide tetraphosphate. The initial nucleotide retains its triphosphate while each added ribonucleotide triphosphate has its two terminal phosphates cleaved as pyrophosphate. The direction of elongation is from 3' hydroxy to 5' phosphate in the DNA, the RNA being made 5' to 3'

in the antiparallel direction. The initial product is complementary to the region of the DNA employed as template and the RNA may be modified post transcriptionally.

Prokaryotic Transcriptions

Background

There are several reviews of prokaryotic transcription available (35,36,56,57) which may be consulted for discussions of aspects that will not be considered here. The basic transcriptional studies have been carried out in bacterial systems with and without phage infection and these studies have served as a model system for understanding the transcriptional process and its control. Only the properties of the bacterial RNA polymerase and the regulation of bacterial RNA synthesis will be reviewed here.

Prokaryotic RNA polymerase

The bacterial RNA polymerases are large molecules (molecular weights between 400,000 and 500,000) and have complex subunit structures. Two enzymatically active forms of the RNA polymerase are currently known: the holoenzyme and the core enzyme. The holoenzyme contains the following polypeptide chain subunits: one beta prime (β') subunit; one beta (β) subunit; two alpha (α) subunits; and either one sigma (σ) subunit or one sigma prime (σ') subunit. The subunits have the molecular weights of approximately 160,000; 155,000; 90,000; and 40,000 respectively. Two forms of the holoenzyme with the structures $\alpha_2\beta\beta'\alpha$(67) and $\alpha_2\beta\beta'\sigma'$(68) have been found in Escherichia coli. The core enzyme lacks a sigma subunit. Therefore, the holoenzyme can be separated into two functional parts: a core enzyme, which is able to synthesize RNA but lacks the

ability to initiate such synthesis specifically; and a sigma subunit, which acts catalytically to allow the efficient initiation of RNA at specific promoter sites. Bacterial RNA polymerases from different species appear to be closely related in subunit structure but show differences in the sizes of the subunits (42). Some preparations of the E. coli enzyme contain a minor component ω (69,70) which has a molecular weight of about 10,000 and is present in the stoichiometry of two ω per holoenzyme. The subunits of the holoenzyme are functional subunits since it has been demonstrated that these subunits are essential for the reconstitution of enzymic activity when the enzyme is reformed from separated subunits (71,72). The ω subunit is not required for reconstitution of RNA polymerase holoenzyme activity.

All transcription in E. coli is sensitive to inhibitors of the purified enzyme, such as rifampicin and streptolydigin (73,74), which block RNA synthesis through interaction with the β subunit of the enzyme (75). Thus, all RNA synthesis appears to depend on an enzyme complex in which the β subunit is functional. The beta subunit is also altered in enzymes from mutants resistant to rifampicin (71) or streptolydigin (76-78).

General mechanism of prokaryotic transcription

Transcription is a process involving a series of different biochemical events. The holoenzyme is able to recognize and to bind to specific regions on the DNA template (the promoters) and to undergo a conformational change, catalyzing the addition of substrate ribonucleoside triphosphates to the binding site (79), with the subsequent formation of the first internucleotide linkage of ATP or GTP with a second

ribonucleoside triphosphate. Inorganic pyrophosphate is eliminated, resulting in the formation of a dinucleotide tetraphosphate of the general structure pppPupX. The sigma subunit appears to be released (80,81) and the core enzyme moves along the DNA template synthesizing the RNA chain by adding ribonucleoside monophosphates to the 3' hydroxy terminus of the nascent RNA chain from ribonucleoside triphosphate substrates. When the RNA polymerase encounters a specific termination site on the DNA template, RNA chain growth is terminated and the nascent RNA chain and the RNA polymerase are released from the template. A termination protein factor, rho, has been implicated in this last step in E. coli (82). However, the RNA polymerase can in some instances terminate accurately without rho.

Prokaryotic polymerases and regulation of transcription

Two basic mechanisms, negative control and positive control, appear to be equally effective in controlling prokaryotic transcription. In negative control, the regulator gene product (a repressor molecule) in its active form binds to the operator gene to prevent the transcription of the operon's structural genes. When the repressor is inactivated by the specific inducer, transcription of the structural genes may proceed. Thus the repressor controls RNA synthesis by negatively affecting RNA polymerase activity with respect to specific sites on DNA. The classic example of negative control is the lac operon of E. coli (83). The lactose operon is under the negative control of the lactose repressor which binds specifically to the lactose operator (84,85) region of the DNA, thus preventing transcription of the DNA past the operator. The expression of this operon requires induction by a galactoside. The inducers bind to the repressor and decrease the repressor's affinity for the lactose operator.

The histidine operon is another example of regulation by negative repression, with histidyl-tRNA as the co-repressor necessary for activation of the histidine repressor which binds to the histidine operator region of the DNA and prevents transcription of the DNA past the operator (86).

In positive control the RNA polymerase has low affinity for the promoter site, and the controlling molecule in its active form helps the RNA polymerase to initiate transcription by increasing the enzyme's affinity for the promotor site. Positive control of gene expression has been demonstrated for the arabinose operon of E. coli (86). The protein product of the C gene is a repressor in the absence of the specific inducer, arabinose. However, in the presence of arabinose the C gene protein is converted to an activator which is required for expression of the arabinose genes.

The sigma subunit of RNA polymerase and the catabolite gene activator protein (CAP) are examples of protein factors which influence transcription by increasing the affinity of the RNA polymerase for promoters. Sigma interacts with the core RNA polymerase and facilitates the binding of the enzyme to a promoter. CAP, also known as cyclic AMP receptor protein, plays a role in the regulation of genes subject to catabolite repression. CAP appears to work in addition to sigma to alter initiation specificity in the presence of cyclic AMP. Cyclic AMP combines with CAP and produces an allosteric change in the protein. The cyclic AMP-CAP complex then binds to the DNA close to the promoter of an inducible operon (i.e., lactose, galactose, tryptophan, and histidine) and produces a change in the DNA so that the RNA polymerase can bind to the specific promoter region and carry out a round of transcription of the operon in question. Thus the expression of the lactose operon requires cyclic AMP, CAP, holoenzyme and the removal of the repressor from the operator.

Alteration of RNA polymerase by phage infection and sporulation

The structure and selectivity of the bacterial RNA polymerase is altered under certain conditions of viral infection and sporulation. The alterations of the selectivity of RNA synthesis occur during lytic growth of the phage in different stages, giving rise to different transcripts during the course of phage replication and assembly. The bacteriophage T_4 infects E. coli and subsequently host RNA synthesis is inhibited, phage-specific macromolecular synthesis is initiated and the host cells are ultimately lysed (87,88). A temporal sequence on phage-specific mRNAs have been detected (89). The first class of mRNA (immediate early) appeared immediately after infection, and hybridized exclusively to one strand of T_4 DNA. The second class of mRNA (delayed early) to appear hybridized to later sequential sites on the same T_4 DNA strand. The late mRNA apparently coded for viral coat protein and it hybridized to the opposite T_4 DNA strand.

The sequence of transcription is mediated by changes in the RNA polymerase. Sigma factor disappears soon after infection (90) and it is this loss which is presumably responsible for restriction of the transcription of the host genome. There is then a sequential alteration of each of the subunits of the core RNA polymerase (26,91,92). The subunit is modified by the covalent attachment of AMP to the alpha subunit. The RNA polymerase acquires four small T_4-specific proteins (93) of 10,000-25,000 daltons which are coded for by the delayed early genes. Thus the transcription specificity appears to be induced by modifications in the polymerase or associated factors.

Bacteriophage lamda can either lysogenize or grow lytically (94,95). During the lytic growth the host E. coli RNA polymerase transcribes the

phage N and Q genes. The two protein products of these genes then mediate the further transcription of the lamda DNA (96). It appears that the N gene product is an antiterminator protein that permits the continued transcription past a rho dependent termination sequence (97). The Q gene product is a repressor that binds to the operator region for the lamda repressor gene, thus preventing the transcription of this gene.

Bacillus subtilis phages SPOL and SP82 show a regulated program of transcriptional alteration during normal growth (98,99). The infected cell RNA polymerase contains two proteins not present in the normal RNA polymerase and the sigma subunit is not required for selectivity (100).

Several observations suggest there are specific changes in the structure of Bacillus subtilis RNA polymerase during sporulation. The purified sporulating RNA polymerase lacks sigma factor and contains a modified β subunit of lower molecular weight than the vegetative enzyme (19,101). In addition, a new polymerase subunit of 60,000-70,000 daltons appears (102). These modifications appear to be due to the action of proteases that are present in cells undergoing sporulation, and it is thought that these changes are related to the alterations in transcriptive specificity that occur during sporulation.

Genetic studies also support a specific role of polymerase in the sporulation process. Mutants which contain an altered beta subunit fail to sporulate (101). The evidence form the genetic studies and the biochemical studies is consistent with the hypothesis that changes in the subunit structure of RNA polymerase mediate the changes in transcription associated with Bacillus subtilis sporulation.

Eukaryotic RNA Polymerases

Background

There are several recent review articles on eukaryotic RNA polymerases (47-52). These may be consulted for discussions that are omitted in this review. The DNA dependent RNA polymerase activity was first demonstrated in rat liver nuclei by Weiss in 1955 (103) who partially purified the enzyme which was firmly attached to DNA. Mans and Novelli (104) were the first to study a solubilized eukaryotic RNA polymerase in 1964. However the problem of freeing the RNA polymerase from DNA was more severe for the other eukaryotic RNA polymerases studied and consequently most studies on nuclear RNA polymerase activity were carried out on isolated nuclei or unpurified chromatin until a eukaryotic RNA polymerase was purified in 1965 (105).

Isolated nuclei demonstrated transcription activity which synthesized mostly GC-rich ribosomal RNA at low ionic strength. This activity was localized in the nuceolus and stimulated by Mg^{++}. However, at high ionic strength mostly DNA-like RNA was synthesized by an activity which was stimulated by Mn^{++} and localized in the nucleoplasm (106-111). The nucleoplasmic activity was specifically inhibited by α-amanitin (3).

Multiple forms of nuclear RNA polymerases were demonstrated by Roeder and Rutter (5) in rat liver and Xenopus laevis. They demonstrated three types of eukaryotic RNA polymerases which could be separated based on chromatographic properties, sensitivity to specific inhibitors and sensitivity to ions. The first RNA polymerase eluted from a DEAE Sephadex column (nuclear RNA polymerase I) was found to be of nucleolar orgin, insensitive to α-amanitin and synthesized a product which hybridized to

RNA competitively with ribosomal RNA (rRNA) but not with heterogeneous RNA (Hn RNA). RNA polymerase II, the second enzyme eluted from the column, was a nucleoplasmic, α-amanitin sensitive RNA polymerase that synthesized a product that hybridized to DNA competitively with Hn RNA but not with r-RNA (32). RNA polymerase III was thought to be nucleoplasmic. Since then several laboratories have demonstrated that the nucleus, nucleolus and cellular organelles have unique transcriptional systems (44).

On a protein percentage basis, the amount of RNA polymerase activity in eukaryotic cells is much lower than in prokaryotes. The RNA polymerases from calf thymus, which is a tissue rich in the enzyme (112), is only obtained in a few milligrams per kilogram of tissue (113-115). This yield, which is a few orders of magnitude lower than that to Escherichia coli RNA polymerase (67,70,116) demonstrates one of the difficulties encountered in studying the regulation of eucaryotic transcription at the molecular level (studies which require significant quantities of the highly purified enzyme). The specific activity of the eukaryotic RNA polymerases is in the order of that of the purified E. coli RNA polymerase (50,113,114,116).

Mitochondrial transcription

The mitochondrion represents a semi-autonomous organelle within the eukaryotic cell in the sense that it contains a unique DNA that is genetically active; it is replicated and transcribed within the organelle to provide products unique from those found elsewhere in the cell. The mitochondrial DNA has been well characterized physically and chemically (117-122) and the size of the mitochondrial genome varies from a molecular weight of 10^7 daltons in vertebrates to 11.9×10^7 daltons in plants (119,122). DNA with these molecular weights can code for only 5,000 amino

acids or about 20 proteins. Therefore, it is impossible for the mitochondrial genome to code for all of the unique mitochondrial proteins which must carry out replicative, transcriptive, translative and metabolic functions unique to the mitochondrion. All available evidence suggests that the mitochondrial DNA carries information for the mitochondrial ribosomal RNA, for at least some mitochondrial tRNAs and for a few mitochondrial membrane proteins (121,123). Thus mitochondrial DNA is required for the biogenesis of mitochondria.

The majority of proteins unique to the mitochondrion are coded for by the nuclear DNA and synthesized on the cytoplasmic ribosomes. Less than 10% of the mitochondrial proteins are synthesized on mitochondrial ribosomes; this fraction consists of probably not more than eight species of hydrophobic proteins that are incorporated into the inner membrane where they assemble cytochromes, cytochrome oxidase and ATPase (120, 121, 123-126). A few components of the mitochondrial protein synthesizing system may be coded for by the mitochondrial DNA, although there is no evidence for the location of the code for the mitochondrial leucyl-tRNA synthetase (127) and the two mitochondrial peptide chain elongation factors, G and T (128). However, the prokaryotic characteristics of the mitochondrial protein synthesizing system makes it difficult to imagine the code coming from elsewhere in the cell.

The only mitochondrial gene products that have been positively identified are ribosomal RNA and transfer RNA (121,123). Hybridization experiments have shown that mitochondrial ribosomal RNA hybridizes specifically with mitochondrial DNA (129-132) and shares no sequence homology between some of the mitochondrial 4s transfer RNA and mitochondrial DNA (133,134). In order to understand the extent to which a mitochondrion depends upon

the rest of the cell, a more complete understanding of the mechanisms which provide unique informational molecules must be examined in detail.

Mitochondrial DNA transcription has been studied *in vivo* using intact growing cells and *in vitro* with isolated mitochondria or solubilized and partially purified mitochondrial RNA polymerase. These investigations have provided results which will be useful for the understanding of the regulatory mechanisms which couple the genetic activities of mitochondria and nuclei during cell growth and development.

The synthesis of mitochondrial RNA in intact cells has been extensively studied in HeLa cells by Attardi and coworkers. Hybridization experiments of *in vivo* labeled mitochondrial RNA with the separated H and L strands of mitochondrial DNA demonstrated that both the H and L strands are equally transcribed but afterwards the product of the L strand is almost completely degraded. This means that the problem of strand selection in mitochondrial transcription could be achieved by transcribing both strands and rapidly degrading or exporting 98% of the L-strand transcripts (135-140). The transcription products have been used to map the mitochondrial DNA; the H-strand contains genes for 9 tRNAs, whereas the L-strand has the two ribosomal RNA genes and also those for three tRNAs (137-141). Election microscopy of ferritin-labeled tRNA-mitochondrial DNA hybrids confirmed the location of these genes (142). Experiments with the HeLa cells also suggest that mitochondrial messenger RNA is also translated. Mitochondrial RNA containing poly (A) hybridizes with mitochondrial DNA (142).

Studies of *in vitro* incorporation of ribonucleotide triphosphates into high molecular weight RNA have shown that isolated mitochondria

are capable, for some time, of carrying out RNA synthesis just as are mitochondria within the living cell, except that the process appears to be much slower *in vitro* than *in vivo*. The incorporation of UTP or ATP generally requires all four ribonucleotide triphosphates and magnesium (143,144). The incorporation process is considerably faster if the mitochondria are swollen. Mitochondrial RNA synthesis is inhibited by actinomycin D, which binds to the DNA template, proving its dependence on DNA. The product of the *in vitro* incorporation reaction hybridizes with the H strand of mitochondrial DNA (145). In *Xenopus* mitochondria the RNA polymerase initiates at several different sites of the template predominantly with ATP and 2-fold less frequently with GTP (146).

Studies on mitochondrial RNA synthesis have recently concentrated on the characterization of the DNA-dependent RNA polymerases. However, relatively little is known about the properties and functions of the mitochondrial enzymes as compared to the nuclear RNA polymerases. It has been difficult to identify and isolate the mitochondrial RNA polymerase due in part to the difficulty of defining proper conditions for the solubilization of this enzyme which appears to be firmly bound to mitochondrial membrane. Studies on mitochondrial RNA polymerases from a variety of sources have shown that these enzymes exhibit properties clearly distinguishing them from the respective nuclear RNA polymerases.

The mitochondrial enzyme differs from the nuclear RNA polymerase II in sensitivity to α-amanitin (11, 147, 148, 149). Low concentrations of α-amanitin (0.1 to 20 μg/ml) which cause inhibition of nuclear RNA polymerase II does not affect the mitochondrial RNA polymerase. Although some functional differences have been described in mitochondrial RNA polymerases, there is general agreement regarding their size.

The molecular weight of the enzyme, consisting of a single polypeptide chain, seems to be about 60,000 (11-15). The purified mitochondrial RNA polymerases from Neurospora crassa (11) and rat liver (12,13,147) are inhibited by rifampicin in contrast to the mitochondrial enzymes from Xenopus laevis (14), wheat leaf (150) and Ehrlic Ascites (39) which are resistant to rifampicin. Some investigators have found the yeast mitochondrial RNA polymerase to be rifampicin resistant (15,148,151-153). Others describe the isolation of rifampicin-sensitive enzyme (154-155). The reason for this discrepancy is not clear. It is possible that a factor or factors needed for conferring sensitivity to rifampicin were lost from some enzyme preparations.

In contrast to the above studies several laboratories have reported that the yeast mitochrondrial RNA polymerases are much larger in size and resemble more closely the nuclear polymerases. Eccleshall and Criddle demonstrated three RNA polymerases associated with yeast mitochondria having molecular weights near 500,000 and showing no sensitivity to rifamycin (153). However, there was no difference between the nuclear RNA polymerase I and the mitochondrial RNA polymerase I, suggesting that their mitochondrial preparation was contaminated with nuclear enzyme. Reports of multiple RNA polymerases associated with yeast mitochondria having molecular weights near 500,000 and showing no sensitivity to rifamycin have come from Rogall and Wintersberger (15) and Benson (156). Scragg has reported a yeast mitochondrial RNA polymerase with molegular weight greater than 200,000 that does show a rifamycin sensitive enzyme in yeast (157). However, despite the low molecular weight (67,000) of the yeast mitochondrial RNA polymerase polypeptide isolated by Rogall and Wintersberger (15), this enzyme readily forms aggregates with molecular

weights up to about 500,000 in buffers of low ionic strength. The same is true for the 64,000 molecular weight enzyme purified by Kuntzel and Schafer (11) and the 60,000 molecular weight enzyme isolated by Scragg (158). The overall picture of mitochondrial RNA polymerase is one of either a great diversity of enzyme types associated with different organisms or else some differences in the isolation procedures employed in the various laboratories which give rise to preparations with these differences. The mitochondrial RNA polymerase consisting of a single polypeptide chain of molecular weight of 64,000 resembles more the bacteriophate T7 specific RNA polymerase rather than E. coli or eukaryotic nuclear RNA polymerase. The T7 enzyme is a product of T7 DNA gene 1. A single polypeptide of 110,000 molecular weight, it generates only eight RNA species in late transcription of T7 DNA (25).

Chloroplast transcription

The chloroplast also contains unique DNA that is replicated and transcribed within the organelle. The molecular weight of Euglena chloroplast DNA is approximately 10^8 daltons (159) which is sufficient to code for the chloroplast tRNA, rRNA, and mRNA. However, the extent to which the total potential genetic content is expressed in chloroplast is open to question. It has been established that the chloroplast DNA contains cistrons coding for chloroplast ribosomal RNA (160,161) and transfer RNA (179). Quantitative saturation-hybridization of Euglena chloroplast rRNA to chloroplast DNA showed that approximately 2% of the DNA is involved in coding for chloroplast rRNA (160). A higher value (6%) was obtained by Stutz and Vandrey (161) who isolated Euglena chloroplast DNA from purified organelles devoid of any nuclear contamination rather

than relying on the fractionation of extracted DNA by preparative CsCl buoyant density centrifugation. The chloroplast ribosomal 23S RNA (1.1×10^6 daltons) and 16S RNA (0.56×10^6 daltons) are coded for by separate cistrons (162) localized on the heavy strand of the chloroplast DNA. Accepting that the molecular weight of Euglena DNA is 10^8 daltons then 2% of this (2×10^6 daltons) is sufficient to code for both the 23S and 16S rRNAs. The value of 6% would allow for three copies of each of the rRNAs per chloroplast DNA molecule.

The presence of mRNA that can be translated has been demonstrated in Euglena chloroplast (163). These mRNAs could be the products of transcription of chloroplast DNA or of nuclear DNA, or of both. However isolated chloroplasts synthesize RNA and such RNA must necessarily be chloroplast-DNA coded. Hybridization of RNA synthesized in isolated tabacco chloroplasts to chloroplast DNA indicated that 21% of the chloroplast DNA is transcribed (164). This represents a much larger proportion of the chloroplast DNA than is required to code only for ribosomal and transfer RNAs and therefore implies that mRNA species are synthesized on chloroplast DNA.

Chloroplasts of higher plants and algae were shown to contain a DNA-dependent RNA polymerase as early as 1964 but the enzyme was only recently solubilized and purified (165). The extreme difficulty with solubilizing this activity was related to the tight binding of the enzyme-DNA complex to the thylakoid membranes. This chloroplast enzyme contained two large polypeptide subunits, which suggested a similarity to the prokaryotic and the eukaryotic nuclear RNA polymerase rather than the mitochondrial or bacteriophage T7 RNA polymerases. The chloroplast enzyme has also been studied in Euglena gracilis (166). The Euglena chloroplast

RNA polymerase has not been solubilized and purified mainly due to the difficulties mentioned above. This prompted the study of chloroplast transcription complexes consisting of chloroplast membrane fragments, DNA, and the RNA polymerase activity.

Euglena RNA polymerases

The nuclear RNA polymerase II of Euglene gracilis was partially purified recently by Congdon and Congdon and Preston (167,168). This enzyme, defined by its sensitivity to α-amantin, eluted from DEAE Sepahdex between 0.18 and 0.21 M $(NH_4)_2SO_4$. The RNA polymerase had a broad salt optimum ranging from 50 mM $(NH_4)_2SO_4$ to 150 mM $(NH_4)_2SO_4$. Optimal activity was exhibited with optimum $MnCl_2$ concentrations rather than with $MgCl_2$. The enzyme was more active with poly d(AT) as a template and preferred denatured over native calf thymus DNA. The Euglena RNA polymerase, as well as the enzymes from other protists, were less sensitive to α-amantin than the corresponding fractions from higher eukaryotes. The Euglena chloroplast and mitochrondrial RNA polymerases have not been previously solubilized and purified.

MATERIALS AND METHODS

Buffers

All buffers were prepared from analytical reagent grade chemicals without further purification. Double distilled or deionized water was used for all solutions. Stock solutions of 1.0 M Tris-HCl pH 7.4, at 25°C, 1.0 M Tris-HCl pH 7.9, at 25°C, 1.0 M Tris-HCl pH 9.0, at 25°C, 0.10 M EDTA pH 7.6, 1.0 M $MgCl_2$, 0.01 M MnCl, 0.10 M dithiothreitol were diluted to prepare the following buffers:

Buffer STE; 0.25 M sucrose, 0.01 M Tris-HCl pH 7.4 or pH 9.0, 0.10 mM EDTA pH 7.6.

Buffer STM; 0.25 M sucrose, 0.01 M Tris-HCl pH 7.9, 0.01 M $MgCl_2$.

Lysis buffer; 0.01 M Tris-HCl pH 7.9,20% w/v sucrose. 0.10 M KCl, 5 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride.

Buffer TGMED; 0.05 M Tris-HCl pH 7.9, 25% glycerol, 5.0 mM $MgCl_2$, 0.10 mM EDTA pH 7.6, 2.0 mM dithiothreitol.

Ethidium bromide buffer; 1 µg/ml Ethidium bromide, 0.01 M Tris-HCl pH 7.9, 0.02 M NaCl, and 0.005 M EDTA.

Organism

Premanently bleached mutants of *Euglena gracilis* klebs strain z, prepared by treatment with streptomycin (169) were used for these studies.

Growth

The streptomycin bleached mutants were cultured in 2800 ml Fernback flasks containing 1500 ml of Medium A described by Greenblatt and Schiff (170).

The cultures were grown at 27° on a gyrotary shaker operating at 120 rev/min. Under these conditions, cells enter stationary phase when the carbon source is exhausted (171). Growth was measured as turbidity with a Klett colorimeter with a No. 66 filter.

Cell disruption and subcellular fractionation

Cells from axenic cultures in exponential phase were harvested by passing the contents of the culture flaks through a DeLaval cream separator centrifuge operating at room temperature. All further operations were performed at 0° -4° C.

The cells were washed twice in STE buffer, pH 7.4, then suspended in at least 5 volumes of STE buffer, pH 9 and passed through an Aminco French Pressure cell at 1000 lb/inch^2 (±100) using a power press to give greater than 90% breakage. Breakage was estimated by microscopic examination. The cell lysate was collected in ice chilled flaks, shakengently to disperse aggregates, and repeatedly centrifuged at 1000 x g for 5 min (Beckman J-21 JA-20 rotor) until only a negligible pellet was produced. Crude subcellular fractions were collected from the cell free lysate supernatant by differential centrifugation first at 5000 x g, 20 min and then 10,000 x g for 15 min. The crude 5000 x g pellet (5P mitochondria) was washed in STE pH 7.4 buffer.

Mitochondrial purification

Washed 5P mitochondria were purified in gradients of sodium diatrizoate (Winthrop Laboratories, New York). Samples containing 5-200 mg dry weight of mitochondria suspended in the less dense renografin solution (density equals 1.103 g/cm^3) containing 0.01 M Tris-Hcl pH 7.4 and 0.10 mM

EDTA were layered over the Renografin gradients (density equals 1.103 to 1.30 g/cm^3) containing 0.01 M Tris-HCl pH 7.4 and 0.01 mM EDTA. The preparations were centrifuged at 20,000 rpm for 2½ hr at 2°C (Beckman L-2, SW 25.1 rotor). Fractions were collected and the mitochondria banding in the region of the gradient corresponding to a density of 1.21 g/cm^3 were washed twice in STE buffer pH 7.4 before use in incorporation studies.

Washed 5P mitochondria were alternatively purified by the enzymatic digestion of nuclear DNA with 50 µg deoxyribonuclease I (E. C. 3.1.4.5) per ml. The crude 5P mitochondria were washed in STM buffer pH 7.9 and then pancreatic DNase I (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 50 µg per ml in STM buffer. Incubation was carried out at 4° for 30 min. with slow stirring. The enzymatic digestion was stopped by the addition of 2 volumes of 10% sucrose, 0.01 M Tris-HCl pH 7.9,0.05 M EDTA pH 7.6. The mitochondria were then washed in STE buffer pH 7.9.

Succinic dehydrogenase assay

Succinic dehydrogenase activity was measured by the succinate dependent reduction of dichlorophenolindophenol (172) in 1 ml reaction mixtures at room temperature.

Extraction of nucleic acids

DNA was extracted from the mitochondria preparations by the technique of Bhargava and Halvorson (173).

Characterization of DNA

The method of Preston and Boone (174) was used for the density measurement of DNA. The CsCl gradient polyacrylamide gels obtained after the

exposure to light were washed with flowing tap distilled water for 2 hr to remove CsCl. The riboflavin was photooxidized by shaking the gels under illumination for 16 hr in the presence of 10 ml of 0.01 N NaOH in 50% formamide. The gels were then washed in tap distilled water for 2 hr before adding the gels to ethidium bromide staining buffer. The staining permitted the quantitative detection of DNA in the gel which could be scanned in an Aminco Flourometer equipped with a gel scanning attachment.

DNA standards

DNA was purified from Cytophage P-11 (a strain isolated, identified and carried at the Department of Microbiology, University of Florida, Gainesville, FL) as described by Preston and Boone (174). DNA from the bacteriophage ϕ25 and DNA from Pseudomonas aeruginosa were kindly provided by M. Mandel, M. D. Anderson Tumor Institute, Houston, Texas. DNA was extracted from isolated nuclei of streptomycin bleached mutants of Euglena gracilis Klebs strain z by the method of Bhargava and Halvorson (173).

Preparation of cellular fractions for protein and DNA analysis

Cellular fraction samples were prepared for analysis by Vortex mixing 1 ml of sample with 5 ml of cold 95% ethanol (-20°C). The particulate material was collected by centrifugation at 4000 rpm, 5 min., 2°C. The ethanol extraction was repeated twice more before suspending the pellet in 1 ml of cold double distilled water. The samples were suspended in 1.0 ml of water then 4.0 ml of cold 0.50 N $HClO_4$ was added and the sample was mixed on a vortex. The precipitated macromolecules were collected by centrifugation before being suspended in 4 ml of cold ether-ethanol (1:3)

and centrifuged again. The pellet was suspended in 4 ml of 0.50 M $HClO_4$ and incubated at 85°C for 20 min. to hydrolyze DNA and RNA, cooled and centrifuged. The top 3 ml of the supernatant was used for DNA analysis. The pellet was suspended in 3 ml of 1.0 N NaOH and heated in a boiling water bath until the precipitated protein was dissolved. This solution was used for analysis of soluble proteins.

Protein determination

The protein concentrations were determined by the Lowry method (175). Dissolved bovine serum albumin served as the standard.

DNA determination

DNA concentrations were determined by the indole method of Hubbard (176). Calf thymus DNA served as a standard. A sample of 0.05 ml was added to 0.05 ml of 10% trichloroacetic acid and 1 ml of 0.04% indole-2 N HCl solution. The mixture was shaken and incubated at 97°C for 15 min. and then cooled in ice. The mixture was then extracted with chloroform three times by adding 2 ml of $CHCl_3$ to the mixture, vortexing and centrifuging 2000 rpm, 5 min. The chloroform phase was removed after each extraction. The absorbance of the aqueous phase at 490 mm was then determined.

RNA polymerase assay

Conditions for the assay of RNA polymerase were similar to those described by Preston et al. (177). The routine mixture contained in a total volume of 0.10 ml: 0.05 M Tris-HCl pH 7.9, 0.005 M $MgCl_2$, 0.001 M $MnCl_2$, 0.001 M DTT, 0.01 M KCl, 0.001 M of each unlabeled ribonucleoside triphosphate, 0.032 mM or 0.004 mM or 0.016 mM (2 Ci/mmole of ^3H-labeled ribonucleoside triphosphate, 100 µg/ml of denatured calf thymus DNA and enzyme. Assay mixtures were incubated for 10 min. (or the time indicated)

at 37°C. The reaction was stopped by adding stop bath (0.05 M sodium pyrophosphate, 2 mg/ml bovine serum albumin, 2 mg/ml Torula RNA and 5 mM of the unlabeled ribonucleotide which was identical to the labeled NTP) and chilling in ice (1). Macromolecules were precipitated by adding 2 ml of cold 10% trichloroacetic acid containing 20 mM sodium pyrophosphate (TCAPP) to each reaction tube. After standing in ice for 15 min. the precipitate was collected by filtration through a glass fiber disc (Whatman GF/C) which had been prewashed with 5 ml of cold TCAPP. Each disc was washed three times with 5-10 ml TCAPP, then three times with 5-10 ml ether-ethanol (1:3) 37°C, followed by two washes 5-10 ml ether. Filtration was carried out in perforated porcelain crucibles (Gooch) adapted to a vaccum manifold with ruber crucible holders (Walter). Filtration was facilitated by maintaining a partial vaccum (ca. 28 in. Hg). The GF/C filters were dried and placed in scintillation vials containing 5 ml of toluene (scintillation grade, Mallinkrodt), 0.4% PPO (2,5-diphenyloxazole), 0.01% POPOP 1,4-di[2-(5-phenyloxazolyl)]-benzene), and counted for 10 min. in a liquid scintillation counter (Beckman LS-133). A unit of enzyme activity is defined as that which will catalyze the incorporation of 1 pmole of labeled ribonucleoside monophosphate into acid precipitable material in 10 min. at 37°.

Isolation of ^{3}H CTP labeled product

A sample of 0.30 ml of purified mitochondria (0.18 mg/ml protein) was incubated in a 3.0 ml RNA polymerase reaction mixture (0.05 mM ^{3}H-CTP, 2 Ci/mmole) for 15 min. The reaction was stopped by adding 3.0 ml of stop bath (0.05 M sodium pyrophosphate, 1.09 mg/ml E. coli RNA and 5 mM of unlabeled CTP) and brought to 0.20 M NaCl and 0.10 M Tris pH9. Triton X

100 was added to bring the mixture to a final concentration of 0.5%. The mixture was then mixed with an equal volume of 80% v/v redistilled aequeous phenol. The nucleic acids were collected by precipitation with 3 volumes of cold 95% ethanol (-20°C) for 12 hr followed by centrifugation at 4000 rpm 15 min. The nucleic acids were dissolved in buffer (0.20 M NaCl, 0.10 M Tris pH 9) and carried through the ethanol precipitation again. The final pellet was dissolved in 2 ml of double distilled water.

Column filtration

Biogel P-4 was swollen overnight by mixing 1 g Biogel per 10 ml of 0.10 M NaCl. A 20 ml column was poured and pre-equilibrated by passing 4 volumes of 0.10 M NaCl through it at 4°C. Nucleic acid samples in 0.10 M NaCl were added to the column and eluted with 0.10 M NaCl. The flow rate was 10 drops per min. ^{3}H labeled samples (100 µl) were placed in 10 ml of Brays scintillation fluid (178) and counted in the liquid scintillation counter (Beckman LS-133).

Extraction of phospholipids

Phospholipid extraction was performed by the method of Carter and Kennedy (179) and Raetz and Kennedy (180). To 0.10 ml of the ^{3}H-CTP labeled product was mixed 1.0 ml of methanol containing 0.10 N HCl. Ten minutes later 2 ml of chloroform was added and the phases were stirred with a vortex mixer before adding 4 ml of 2 M KCl. The mixture was then vigorously mixed for 5 min. and the phases were allowed to separate before the aqueous phase was removed. The chloroform phase was washed two more times with 2 M KCl. A sample of each phase following extraction was placed in a scintillation vial and dried by passing a stream of nitrogen

gas over the solution. Then 10 ml of Bray's solution was added to the vials and counted.

Thin layer chromatography methods

The procedure of Ter Schegget et al. (181) for thin layer chromatography of CDP-diglyceride was followed. Samples were spottedon silica gel G thin layer plates. Chromatograms were developed with a solvent of chloroform-methanol-water-ammonia (70:38:2:8). The CDP-diglyceride standard was prepared by the method of Carter and Kennedy (179). Nucleotides were chromatographed on PEI cellulose by the methods of Randerath and Randerath (182,183). Ribonucleoside monophosphates were chromatographed first in 1.0 N acetic acid to a height of 4 cm and then without drying the plate was transferred to a tank containing 0.30 N LiCl and the solvent was allowed to rise to 15 cm. The ribonucleoside triphosphates were separated by developing to 15 cm with 0.50 M ammonium sulfate.

Solubilization and purification of mitochondrial RNA polymerase

Solubilization of mitochondrial RNA polymerase was achieved by following a procedure similar to that of Wintersberger and Rogall (15, 152). Mitochondria from 150 g wet weight of cells were suspended in an equal volume of lysis buffer. This was added to an equal volume of a lysis mixture consisting of 1.6% Triton X 100 non ionic detergent, 1.0 M KCl, and 0.01 M EDTA (pH 7.9). The mixture was mixed gently for 15 min and then centrifuged for 90 min at 30,000 rmp (Ti 50 rotor) and 0.29 g of solid ammonium sulfate per ml of 30,000 supernatant was added to bring the extract to 50% saturation with respect to ammonium sulfate. The precipitate was collected by centrifugation at 40,000 rmp for 90 min.

(Ti 50 rotor) and dissolved in TGMED containing 0.05 M $(NH_4)_2SO_4$ and dialyzed against 2 liters of the same buffer for 4 hr. The dialyzate was applied to a column (0.90 x 20 cm) of DEAE-Sephadex A-25 which had previously been equilibrated with the same buffer and nonabsorbed protein was washed off the column with 30 ml of the same buffer. The RNA polymerase activity was eluted with a 160 ml gradient of 0.05 M to 0.50 M $(NH_4)_2SO_4$ in TG,ED. Fractions (2.50 ml) were collected and 20 µl of each fraction was assayed for RNA polymerase activity. Concentrations of $(NH_4)_2SO_4$ were determined with a conductivity bridge.

Chemicals and reagents

The following research grade biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo: The ribonucleoside triphosphates GTP, CTP UTP and ATP as sodium salts; dithiothreitol; phenylmethylsulfonyl flouride; high molecular weight calf thymus DNA; Torula yeast RNA, Triton X-100; enzyme grade trizma buffers; chromatographically pure pancreatic ribonuclease A (EC 2.7.7.16) and deoxyribonuclease I (EC 3.1.4.5): fraction V bovine serum albumin; cytidylyl (3'→ 5') cytidine (C_pC); and snake venom phosphodiesterase (Crotalus atrox venom, Type VII).

The tridium labeled ribonucleoside triphosphates as sodium salts were purchased from Schwartz/Mann, Orangeburg, NY. These include {5-^{3}H} UTP, {5-^{3}H} CTP, {8-^{3}H} ATP, {8-^{3}H} GTP. Ethidium bromide and actinomycin D were purchased from Calibiochem, LaJolla, CA. Poly d(AT) was obtained from Miles Laboratories, Kankakee, IL. The rifamycin derivativies AF/05 and AF/013 were the generous gift of Gruppo Lepetit, Milan, Italy. The α-amanitin was kindly provided by Dr. T. Wieldand, Max-Planck Institute,

Heidelberg, West Germany. Sodium diatrizoate was purchased from Winthrop Laboratories, NY. Nonionic detergent NP-40 was purchased from Shell Oil Co. Unless specifically noted, all other chemicals were analytical reagent grade.

RESULTS

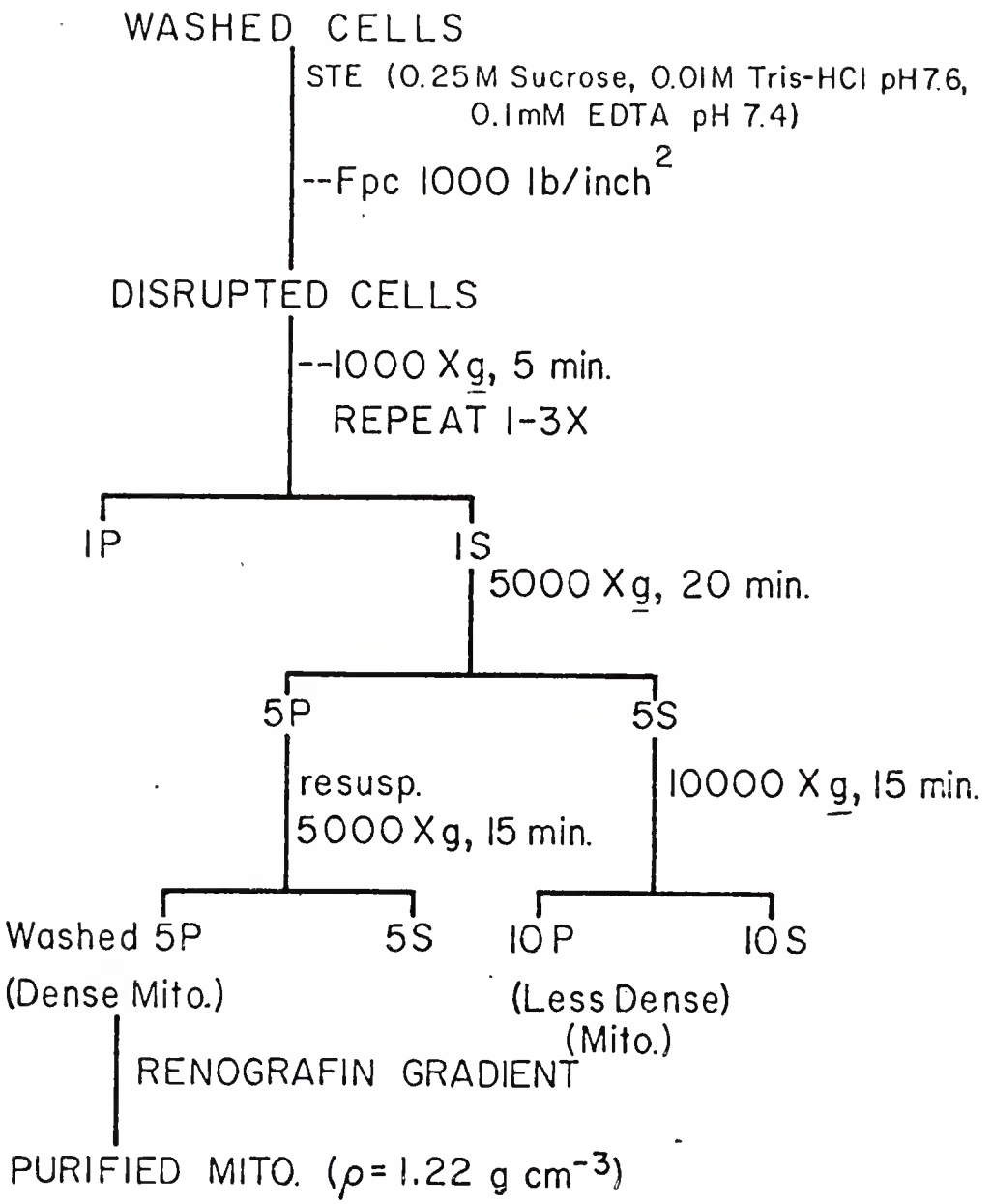
Two experimental approaches were utilized to study the *in vitro* mitochondrial RNA synthesis in *Euglena gracilis*. The first approach was to study the incorporation of radioactively labeled ribonucleoside triphosphates into acid insoluble products by isolated mitochondria which had been treated with isotonic buffer to assure swelling and permability. The products of the incorporations were then studied. The second approach was to study the activity of solubilized and partially purified mitochondrial DNA dependent RNA polymerase. This approach involved determining the conditions for solubilizing the enzyme and then for partially purifying the enzyme. The activity of this enzyme was then characterized.

The Incorporation of Ribonucleoside Triphosphates by Isolated Mitochondria

The initial research objectives were to determine if RNA synthesizing activity is retained by mitochondria isolated from *Euglena gracilis*, and to look for ways to differentiate between nuclear and mitochondrial RNA polymerase activities such as sensitivities to inhibitors or special enzymatic requirements. The approach was to study the RNA synthesizing activity of isolated mitochondria obtained from a streptomycin bleached aplastidic mutant of *Euglena gracilis*, strain z. The mitochondrial isolation procedure (Figure 1) consisted of rupturing washed cells in the French pressure cell at 1000 lb/in^2 and collecting crude mitochondrial

Figure 1. SCHEMA FOR THE ISOLATION OF MITOCHONDRIA

ISOLATION PROCEDURE

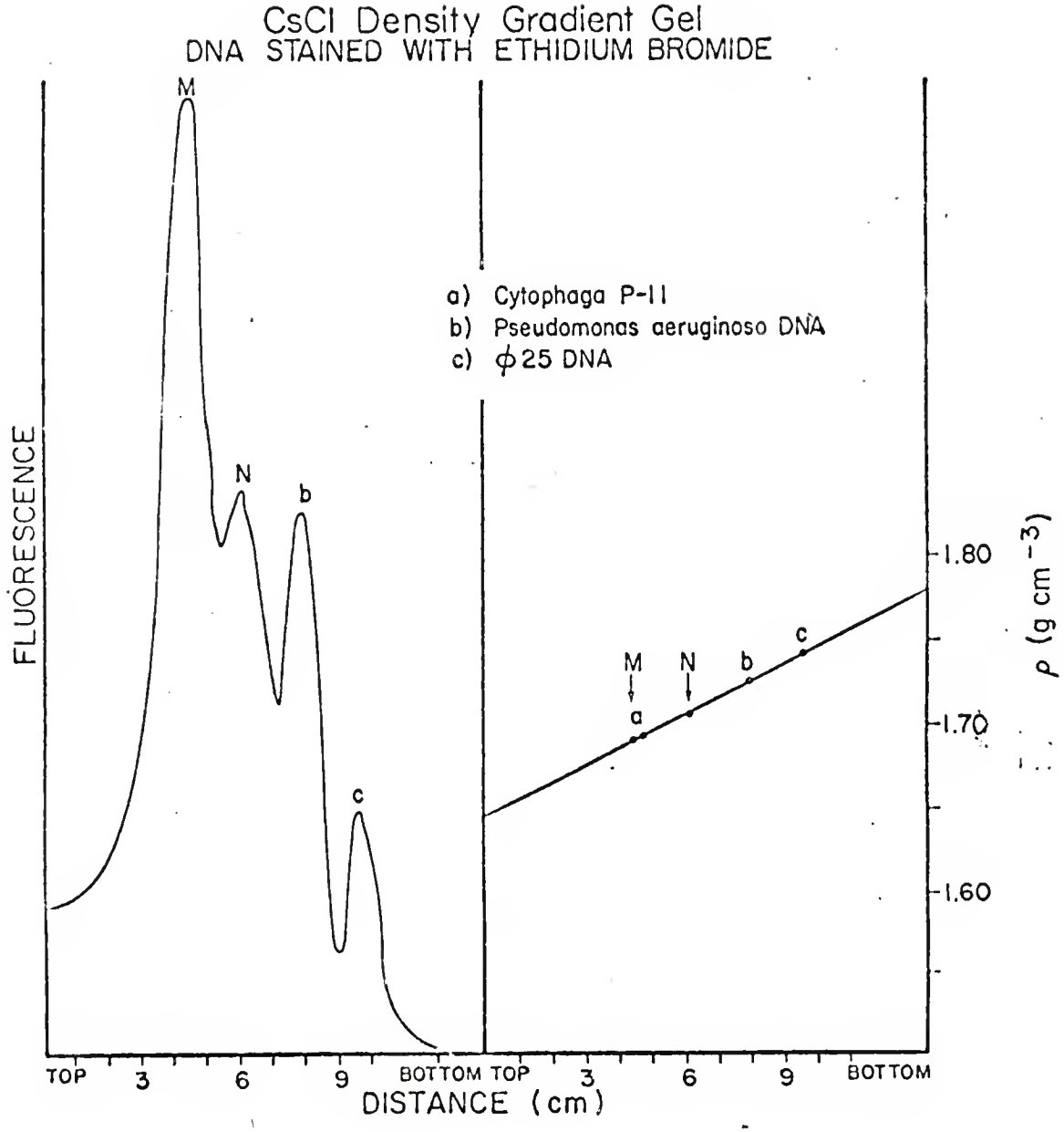


pellets by differential centrifugation, first at 5000 x g and then at 10,000 x g for 15 min. The crude 5000 x g mitochondrial pellet (5P) containing the heavier and more enzymatically active mitochondria (33) were used for these experiments. The 5P mitochondria were washed in buffer and then subjected to sodium diatrizoate (renografin) gradient centrifugation at 20,000 x g for 2½ hr. Fractions were collected and the mitochondria, which banded in the region of the gradient corresponding to a mean equilibrium density of 1.22 g/cm^3 were used for the studies reported here.

In order to test the purity of the mitochondrial preparation, the buoyant density of the DNA in the mitochondrial preparations was routinely monitored by CsCl gradient centrifugation. The CsCl gradients were generated in the presence of acrylamide and catalyst according to the procedure of Preston and Boone (174). This method allows the DNA that is banded in the CsCl gradient to be fixed by exposing the gradient to light in order to polymerize the acrylamide gels. Ethidium bromide staining permitted a rapid and sensitive technique to quantitatively detect the DNA in the gels which could be scanned in an Aminco Fluorometer equipped with a gel scanning attachment. The linear relationship between the relative position in the gradient and the buoyant density of the DNA is demonstrated by the plot of DNA standards of known densities in Figure 2. The purified mitochondrial preparation yields better than 70% mitochondrial DNA which has a density of 1.691 g/cm^3 and less than 30% nuclear DNA which has a density of 1.707 g/cm^3. Although it is evident that the preparation contains some contaminating nuclear DNA, this method provided the cleanest feasible mitochondria without treating the mitochondria with an exogenous nuclease.

Figure 2. DENSITY EQUILIBRIA ANALYSIS OF MITOCHONDRIAL DNA

Density equilibria anlaysis of DNA in mitochondria purified on a sodium diatrizoate (Renografin) gradient were measured by CsCl density gradient centrifugation as described in the methods section. The markers are Cytophage P-11 DNA with a density of 1.693 g/cm^3, Psuedomonas aeruginosa DNA with a density of 1.727 g/cm^3, and bacteriophage ϕ DNA with a density of 1.742 g/cm^3.


CsCl Density Gradient Gel
DNA STAINED WITH ETHIDIUM BROMIDE
a) Cytophaga P-11
b) Pseudomonas aeruginoso DNA
c) ϕ25 DNA
FLUORESCENCE
M
N
b
c
TOP
3
6
9
BOTTOM
TOP
3
6
9
BOTTOM
DISTANCE (cm)
M
N
b
c
a
1.80
1.70
1.60
ρ (g cm⁻³)

The renografin purified mitochondria are active biochemically as demonstrated by results in Table 1. Succinic dehydrogenase which is tightly integrated into the inner membrane of mitochondria has been employed as a marker enzyme in order to follow the purification of mitochondria. Of the total cellular succinic dehydrogenase activity 42% is recovered in the purified mitochondria, with a 27-fold purification per mg of protein and a 70-fold purification per µg of DNA. This demonstrates that the purification procedure yields mitochondria biochemically active for an enzyme not associated with the DNA

A Mitochondrial Activity Incorporating Label From ^{3}H-CTP Into Acid Precipitable Product

The incorporation of label from the four ribonucleoside triphosphates labeled with tritium by isolated mitochondria was studied by performing RNA polymerase assays as described in the methods section. The mitochondria were treated with isotonic buffer to assure swelling and permability. The relative rate with which label from each ribonucleoside triphosphate was incorporated into acid insoluble macromolecules was determined by performing the assays in 0.1 ml reaction mixtures in which only one of the ribonucleotides was labeled. It was observed that isolated mitochondria catalyzed the incorporation of label from ^{3}H-CTP into acid insoluble material at a rate greater than 10 times that for the other ribonucleotides (Table 2). It is interesting that ATP and GTP, the ribonucleotides which normally serve as the best substrate for RNA initiation in procaryotic systems, are the poorest substrates for the reactions studied here. Figure 3 demonstrates the relative rates of incorporation of tritium from ^{3}H-CTP and ^{3}H-UTP, the two most active substrates. In this experiment the concentration of

TABLE 1

DISTRIBUTION OF SUCCINIC DEHYDRAGENASE ACTIVITY

FRACTION	TOTAL UNITS	PERCENT RECOVERY	UNITS/mg PROTEIN, (X 10^{-4})	FOLD PURIFICATION	UNITS/µg DNA, (X 10^{-4})	FOLD PURIFICATION
LYSATE	1.96	100	7	1.0	0.2	1.0
5P MITO	1.78	90	59	8.4	1.1	7.5
WASHED 5P MITO	0.91	46	65	9.3	1.1	7.6
RENO MITO	0.82	42	190	27.0	10.3	70.0

Succinic dehydrogenase activity was measured by the succinate dependent reduction of dichlorphendol-indophenol (160) in 1 ml reaction mixtures at room temperature. The protein and DNA concentrations were determined by the procedures described in the methods and procedures. A unit of succinic dehydrogenas activity is defined as a µmole of dichlorophenolindophenol reduced per minute per ml of enzyme sample at 25°C.

TABLE 2

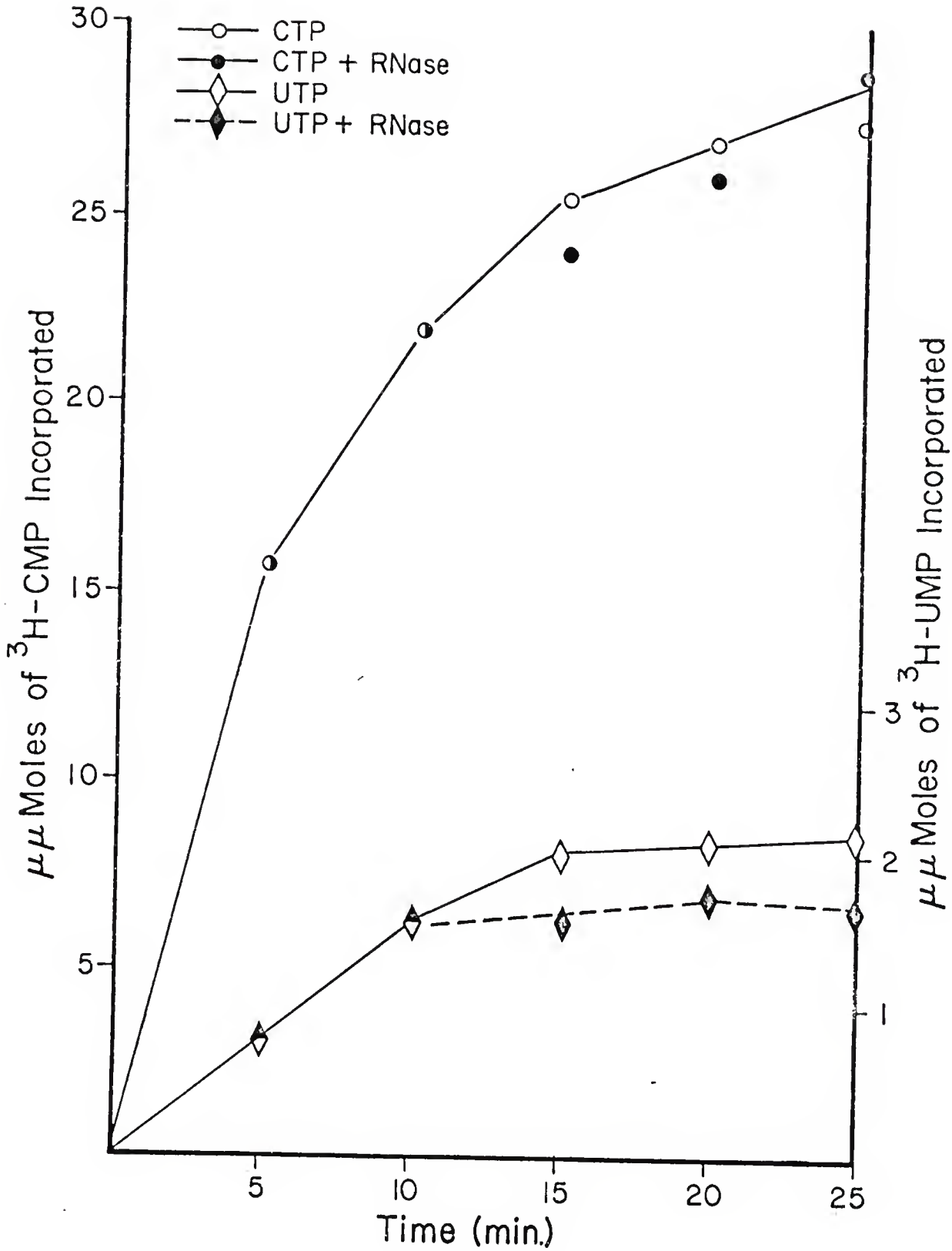
NUCLEOTIDE INCORPORATION BY
ISOLATED MITOCHONDRIA

Labeled Ribonucleotide	CPM incorporated into acid insoluble macromolecules.
^{3}H-CTP	13860
^{3}H-UTP	952
^{3}H-ATP	276
^{3}H-CTP	322

The RNA polymerase assay is described in the methods section. Each reaction mixture contained in a total volume of 0.1 ml: 1.0 mM of each unlabeled NTP and 0.004 mM of each ^{3}H-labeled ribonucleoside triphosphate with a specific radioactivity of 2 Ci/mmole.

Figure 3. RIBONUCLEOSIDE TRIPHOSPHATES INCORPORATION BY ISOLATED MITOCHONDRIA

The incorporation of ribonucleoside triphosphates by isolated mitochondria into acid insoluble products. The RNA polymerase assay described in the methods section was followed except that the concentration of the radioactively labeled ribonucleoside triphosphates was 0.016 mM, 2 Ci/mmole. The unlabeled ribonucleotides concentration was 0.5 mM each.


CTP
CTP + RNase
UTP
UTP + RNase
30
25
20
15
10
5
μμ Moles of ³H-CMP Incorporated
3
2
1
μμ Moles of ³H-UMP Incorporated
5
10
15
20
25
Time (min.)

the radioactively labeled ribonucleotide was 0.016 mM and that of the unlabeled ribonucleotides was 0.5 mM each. The other components of the reaction were as described in the methods. Label from ^{3}H-CTP was incorporated at a rate greater than 10-fold that for UTP. It should be noted that the CTP and UTP tritium incorporations are plotted on different scales in Figure 3. When the incubation is carried out in the presence of RNase A, the product formed with ^{3}H-CTP incorporation shows no sensitivity whereas the product formed with ^{3}H-UTP shows some sensitivity to RNase A. This could be due to the possibility that the CTP product is not RNA or that the product is complexed with DNA or folded and complexed such that it is RNase insensitive. These results also suggest that RNA synthesis could be better followed by studying the incorporation with labeled UTP.

The incorporation of tritium from ^{3}H-CTP by the isolated mitochondria was clearly not dependent upon the presence of the other ribonucleoside triphosphates which appear to inhibit the incorporation by about 10 percent (Table 3). Incorporation of label from ^{3}H-UTP also shows no dependence upon added ribonucleotides while the cpm for ATP and GTP were too low to make any judgment as to their dependency.

The high rate of incorporation with ^{3}H-CTP relative to that with the other ribonucleoside triphosphates prompted the investigation of the incorporation with ^{3}H-CTP. This activity demonstrated no dependency upon added exogenous DNA or ribonucleoside triphosphates (Table 4). In fact the isolated mitochondria incorporated label from ^{3}H-CTP at a 10 times greater rate in the absence of added DNA or nucleoside triphosphates than when they were present.

The distribution of the incorporating activity with ^{3}H-CTP was followed through the isolation procedure and the results are shown in Table 5. It was

TABLE 3

INCORPORATION OF RIBONUCLEOSIDE TRIPHOSPHATES BY ISOLATED MITOCHONDRIA.

Labeled Ribonucleotide	CPM/Rx.Mix.	Units/g Wet Wt.
^{3}H-CTP		
+ NTPs	11,513	2161
- NTPs	12,792	2401
^{3}H-UTP		
+ NTPs	616	116
- NTPs	1,386	260
^{3}H-ATP		
+ NTPs	206	39
- NTPs	324	61
^{3}H-GTP		
+ NTPs	84	16
- NTPs	290	54

The RNA polymerase assay was performed as described in the methods. 0.1 ml reaction mixtures contained 1.0 mM of each unlabeled NTP, 0.004 mM ^{3}H NTP, 2 mC/mmole.

TABLE 4

THE DEPENDENCY OF THE MITOCHONDRIAL ACTIVITY INCORPORATING LABEL FROM ^{3}H-CTP UPON ADDED RIBONUCLEOTIDES AND DNA

Assay Components	Units/mg Protein
Complete Assay with DNA	1,500
-DNA + NTP	1,481
-DNA - NTP	11,056
-DNA + UTP	9,611
-DNA + ATP	10,667
-DNA + GTP	8,611

The RNA polymerase assay was performed as described in the methods. 0.1 ml reaction mixtures contained 1.0 mM of each unlabeled NTP and 0.004 mM of ^{3}H-CTP with a specific radioactivity of 2 Ci/mmole.

TABLE 5

DISTRIBUTION OF ^{3}H-CMP INCORPORATING ACTIVITY

Fraction	TOTAL Units	% Recovery	Units/mg Protein, X 10^2	Fold Purification	Units/µg DNA	Fold Purification
Lystate	141,750	-	2.0	-	0.9	-
5P Mito	39,420	28	6.5	3.2	6.5	7.1
Washed 5P Mito	12,320	9	5.9	3.0	7.7	8.0
Reno Mito	18,940	13	17.4	8.7	114.0	125.0

The RNA polymerase assay is described in the methods. Each reaction mixture contained in a total volume of 0.1 ml; 1.0 mM of each unlabeled NTP and 0.004 mM of ^{3}H-CTP with a specific radioactivity of 2 Ci/mmole.

observed that 13% of the activity was recovered in the purified mitochondria with an 8.7-fold purification per mg of protein and 125-fold per µg of DNA. This suggests that if the activity is associated with DNA, then it is with mitochondrial DNA and not nuclear DNA, 95% of which has been removed by the purification procedure.

The incorporation of ^{3}H-CTP into acid insoluble product is inhibited by actinomycin D (Table 6) at concentrations above 50 µg per ml, suggesting that a DNA dependent reaction may be involved. However, it is interesting that relatively high concentrations are required to demonstrate this inhibition. The possibility of a secondary site of action of actinomycin D or inhibition by other compounds in the actinomycin D can not be ruled out. However, pyrophosphate at 2 µmoles per ml inhibited the activity while inorganic phosphate at 2 µmoles per ml failed to inhibit the incorporation indicating that the incorporation involves a pyrophosphorolysis. This indicates the incorporation of CMP and rules against reactions which incorporate CMP from CDP such as that of nucleotide phosphorylase or the incorporation of CDP from CTP.

Rifampin and streptovaricin, inhibitors of initiation and elongation of prokaryotic RNA polymerases respectively, failed to inhibit the activity. Rifamycin AF/013, and inhibitor of initiation with eukaroytic nuclear RNA polymerases as well as prokaryotic RNA polymerases, also failed to inhibit the activity. Ethidium bromide, which will bind to DNA, showed only slight inhibition which is probably not significant.

The acid insoluble product is sensitive to snake venom phosphodiesterase and alkaline hydrolysis (Table 7) under two different conditions, suggesting that the product contains phosphodiester bonds. Up to this point the product had demonstrated properties that were similar to those

TABLE 6

INHIBITION OF ^{3}H-CMP INCORPORATION

Addition	CPM	Percent of Control
H_2O	4213	100
1% DMF	4179	100
Actinomycin D, 100 µg/ml	602	14
Actinomycin D, 75 µg/ml	2052	48
Actinomycin D, 50 µg/ml	4708	100
Pyrophosphate, 2 µmoles/ml	456	10
Phosphate, 2 µmoles/ml	4055	96
Rifampin, 100 µg/ml	3538	84
Rifamycin AF/013, 100 µg/ml	4050	96
Ethidium Bromide, 100 µg/ml	3370	80
Streptovaricin, 100 µg/ml	3918	93

The RNA polymerase assay was performed with the addition of inhibitors to the final concentrations indicated above. Rifampin, rifamycin AF/013, ethidium bromide and streptovaricin were solubilized in 1% N'N' dimethyl formamide.

TABLE 7

CHARACTERIZATION OF PRODUCT

Addition	CPM	Percent of Control
Control	6412	100
Snake Venom Phosphodiesterase, 10 µg/ml	561	9
0.1M KOH 100°C, 20 min.	154	2
H_2O, 100°C, 20 min.	4437	70
0.5M KOH, 37°C, 16 hr.	119	2
H_2O, 37°C, 16 hr.	5129	80

The RNA polymerase assay was performed with the addition of phosphodiesterase (E. C.: 3.1.4.1) from Crotalus atrox venom, Type VII (Sigma, St. Louis). The alkaline hydrolysis was performed by first performing the RNA polymerase assay for 10 minutes and then treating the reaction mixture with KOH for the indicated times before the addition of stop bath and the precipitation of macromolecules with 10% trichloroachetic acid.

reported by Duda and Cherry (184) for a poly C polymerase activity in sugar beet nuclei.

The RNA polymerase assay reaction mixture was scaled up so that the labeled products of the incorporation of ^{3}H-CMP could be studied. For this purpose the reaction mixture contained in a total volume of 3.0 ml, 5.0 mM of each unlabeled ribonucleoside triphosphate and 0.05 mM of ^{3}H-CTP with a specific radioactivity of 2 Ci/mmold. After incubationof 15 min the products were isolated as described in the methods and material section by phenol extraction followed by ethanol precipitation of labeled products from the aqueous phase in the presence of carrier E. coli RNA and CTP. Zonal centrifugation (Figure 4) on sucrose gradient was performed in order to determine the size of the tritium labeled product. The radioactivity remained at the top of the gradient indicating the material was smaller than that of E. coli 5S RNA. Therefore, the sizes of the isolated products were estimated by Biogel P-4 column filtration (Figure 5). The E. coli carrier RNA eluted in the void volume while two adsorbancy peaks were eluted which contained the labeled cytosine. Using the elution position of a standard C_pC the molecular weight of the first peak (BGP-1) was estimated to be approximately 800 which corresponds to the molecular weight of a trinucleotide of poly C. The molecular weight of the second peak (BGP-2) was estimated to be about 680 which corresponds to the molecular weight of a dinucleotide. It should be noted that this smaller entity elutes before C_pC indicating that it is larger than a dinucleotide of this structure.

The components in the two peaks eluted from the P-4 column have ultraviolet absorbancy spectrums very similar if not identical to that of C_pC, showing the characteristic absorption maximum and minimum at

Figure 4. SUCROSE GRADIENT ZONAL CENTRIFUGATION

The ^{3}H-CMP-labeled product was isolated from the scaled up RNA polymerase reaction mixture using carrier E. coli RNA. The tritium-labeled materials (200 µl) obtained from the nucleic acid extraction of the scaled up reaction mixture were layered over 5 ml sucrose (Schwarz Mann special density grade) gradients, 5 - 20% w/v, containing 0.01M Tris-HCl pH 7.6 and 0.02 M NaCl and centrifuged (SW 39 rotor) 6 hr at 4^{o}C. The E. coli RNA standard was centrifuged in a separate tube. The gradient was analyzed by pumping the gradient through the Gilford J cell, delivering the densest portion first with a bottom probe. The ultraviolet absorbancy was monitored with the Gilford recorder. Fractions were collected and measured for radioactivity by placing 200 µl samples in Brays scintillation fluid and counted in the scintillation counter. The ultra-violet absorbancy profiles for the products (————) and E. coli RNA (————) and the radioactivity profile (—·—·—) are shown

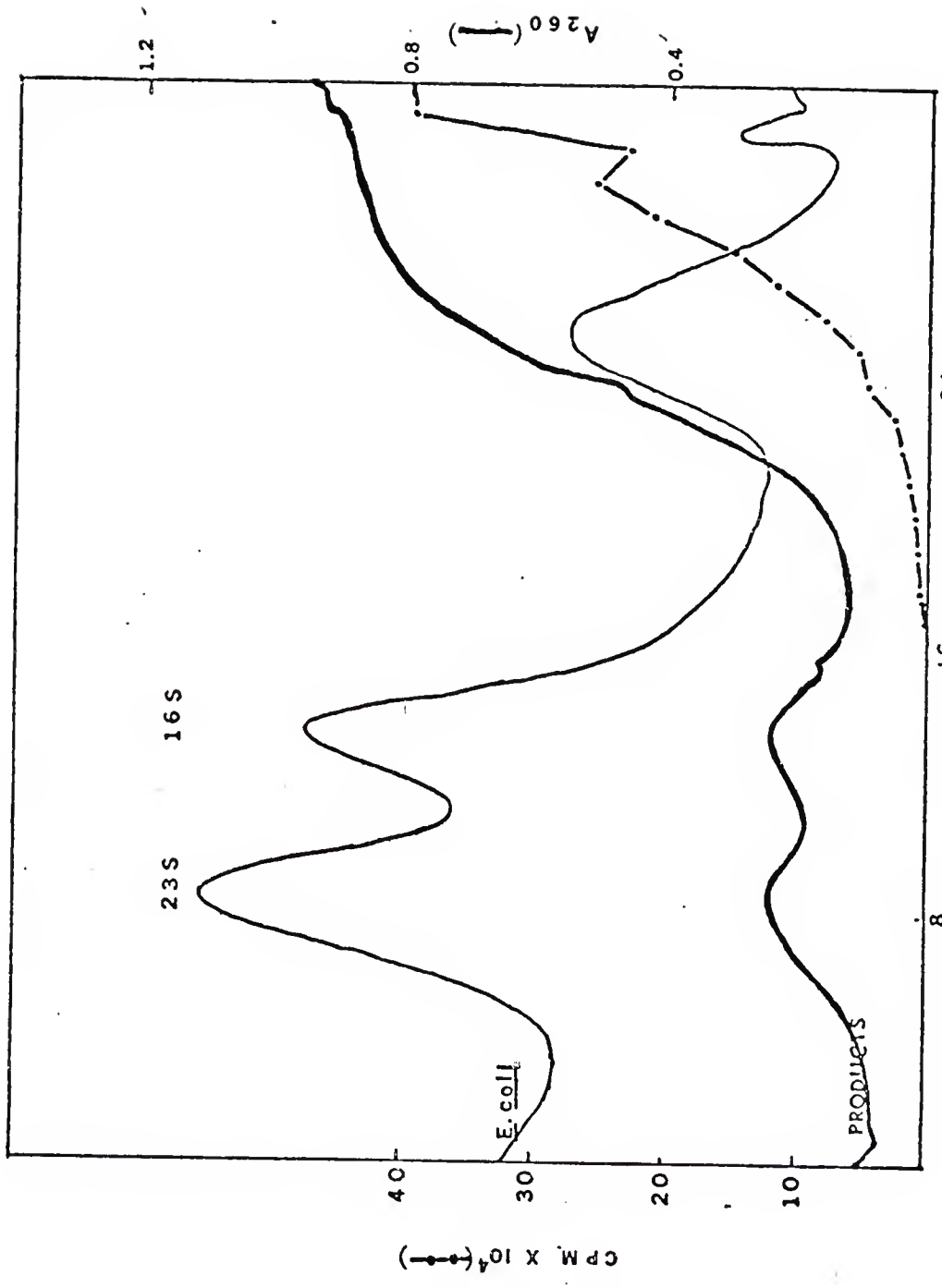
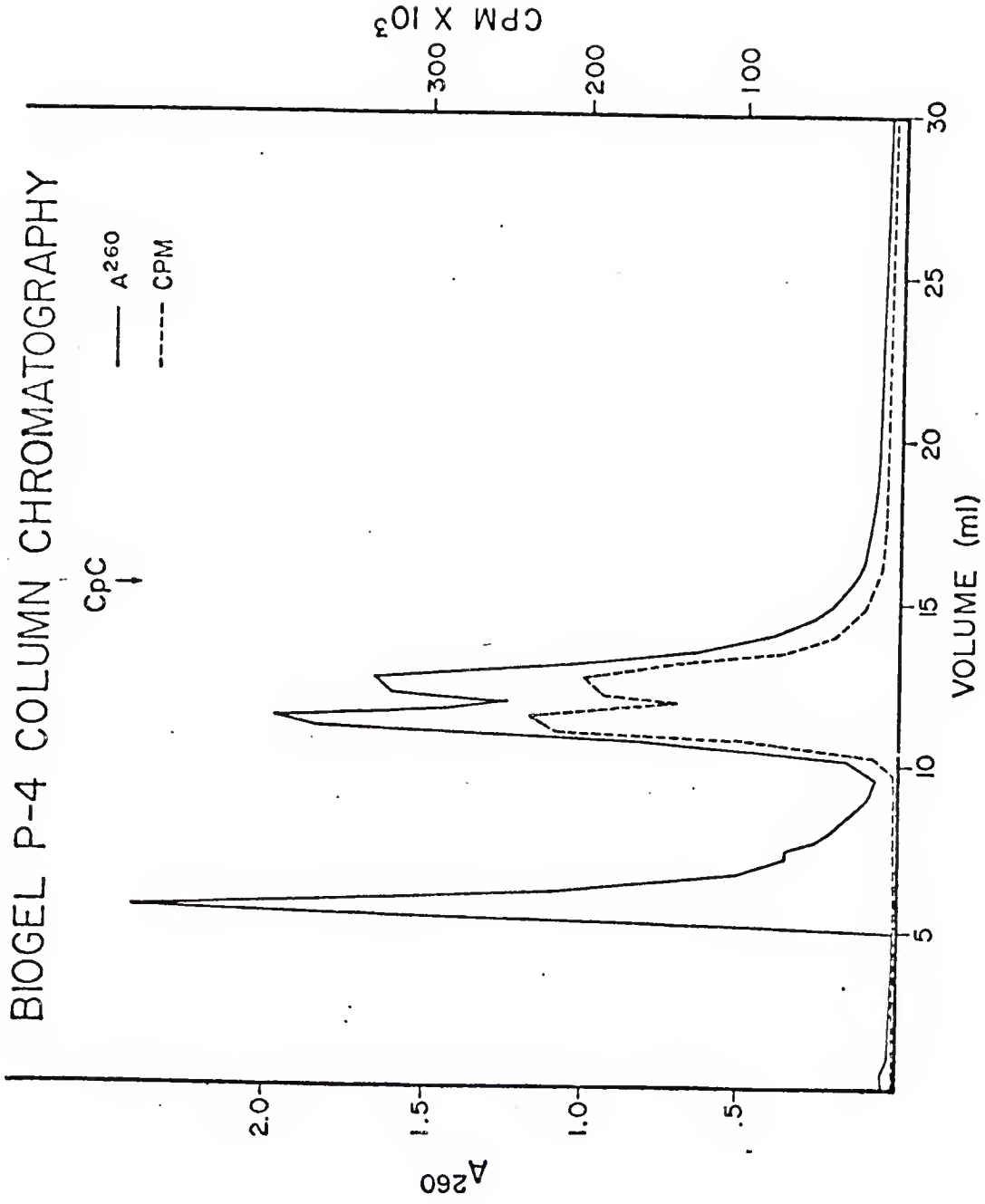

CPM. X 10⁴ (—●—)
40
30
20
10
E. coli
PRODUCERS
23S
16S
A260 (——)
1.2
0.8
0.4
8
16
24
FRACTION NUMBER

Figure 5. BIOGEL P-4 COLUMN CHROMATOGRAPHY

The ^{3}H-CMP labeled product was isolated from the RNA polymerase reaction mixture using carrier E. coli RNA. The product samples dissolved in 0.1 M NaCl were added to a 20 ml Biogel P-4 column previously equilibrated with 0.1 M NaCl. Fractions were eluted with 0.1 M NaCl at 4^{o}C. The eluted fractions were assayed for radioactivity. The standard was $C_{p}C$.


BIOGEL P-4 COLUMN CHROMATOGRAPHY
CpC
A260
CPM
A260
2.0
1.5
1.0
.5
CPM X 10³
300
200
100
5
10
15
20
25
30
VOLUME (ml)

pH 2 and pH 7 for cytosine (Figure 6). This demonstrates that cytosine is the only base incorporated into the products, and suggests that the products are homopolymers of CMP.

The possibility that the product may be CDP-diglyceride was investigated, since it has been reported that crude mitochondrial preparations from rat liver incorporated label from both CTP and dCTP to CDP diglyceride and dCTP diglyceride respectively (181). These mitochondria incorporated label from ^{3}H-dCTP into acid-insoluble material at a much higher rate than any other deoxyribonucleoside triphosphate. About 95% of the acid-insoluble material labeled after incubation with ^{3}H-dCTP could be extracted by chloroform. Therefore, studies were performed to ascertain if Euglena mitochondria incorporated the label from ^{3}H-CTP into CDP-diglyceride.

The results of phospholipid extraction are shown in Table 8. A 0.1 ml sample of the ^{3}H-CMP labeled product obtained after nucleic acid extraction and dissolved in water was mixed with 1.0 ml of methanol for 10 minutes before adding 2.0 ml of chloroform with additional mixing. Then 4.0 ml of 2 M KCl was added with vigorous mixing (Vortex mixer, 5 min., 25^{o}C). After the phases separated upon standing, less than 1% of the tritium label could be accounted for in the chloroform phase whereas 64% of the label could be accounted for in the aqueous phase. Repeated additions of the aqueous KCl to the chloroform phase resulted in no extraction of additional label into the aqueous phase. The difficulty in accounting for all of the radioactivity was due to the quencing produced by the aqueous KCl phase in the Bray's scintillation fluid.

Further evidence that the product is not CDP-diglyceride was provided by the failure of the radioactively labeled material eluted from the P-4 column to migrate in a silica gel G TLC system (Figure 7) in

Figure 6. ULTRA-VIOLET ABSORBANCY SPECTRA

The ultraviolet absorbancy spectra of the ^{3}H-CMP labeled products eluted from the Biogel P-4 column were performed after adjusting the pH. The spectrum for the standard, C_pC, was the same as before and after column filtrations.

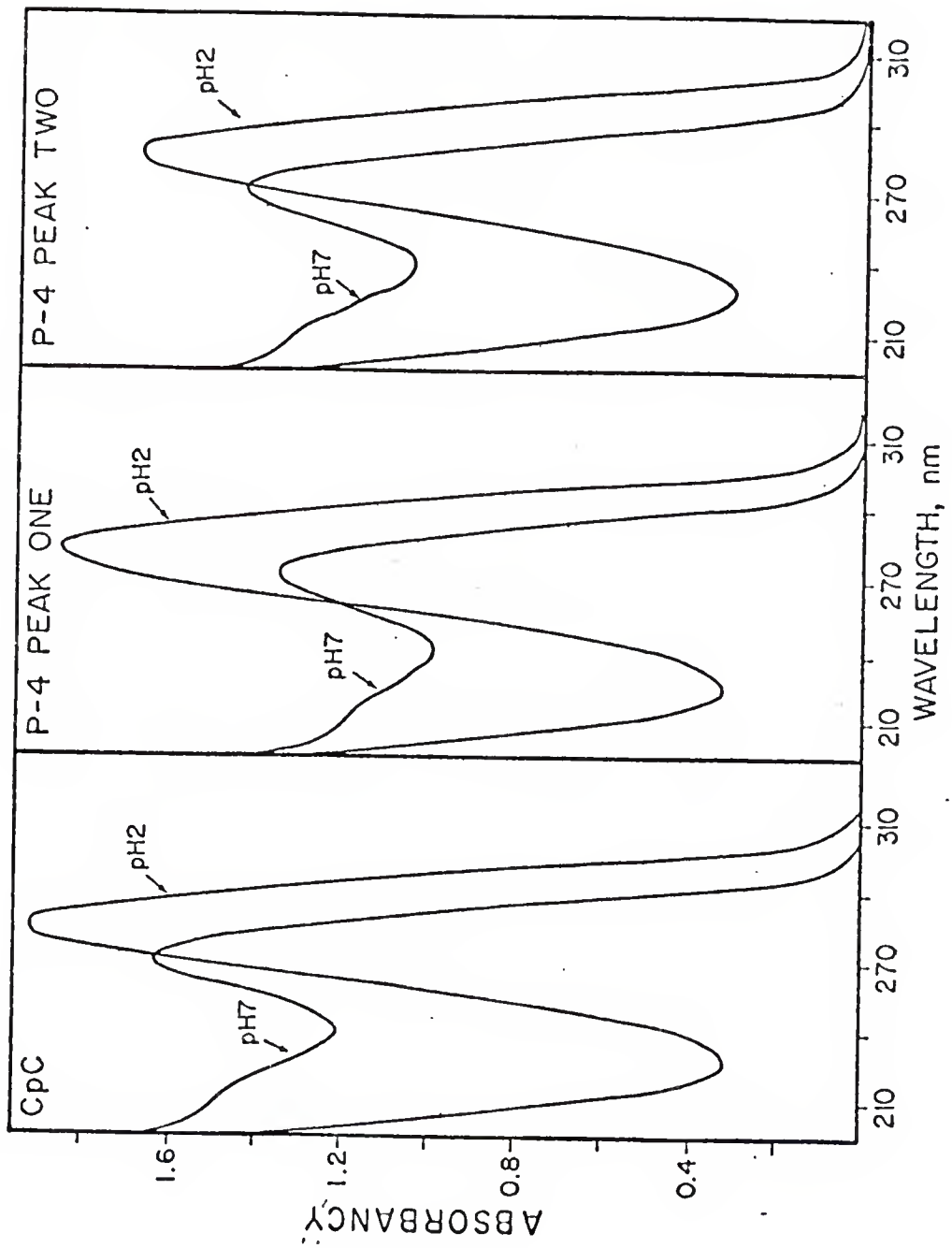

CpC
P-4 PEAK ONE
P-4 PEAK TWO
pH2
pH7
ABSORBANCY
1.6
1.2
0.8
0.4
210
270
310
WAVELENGTH, nm

TABLE 8

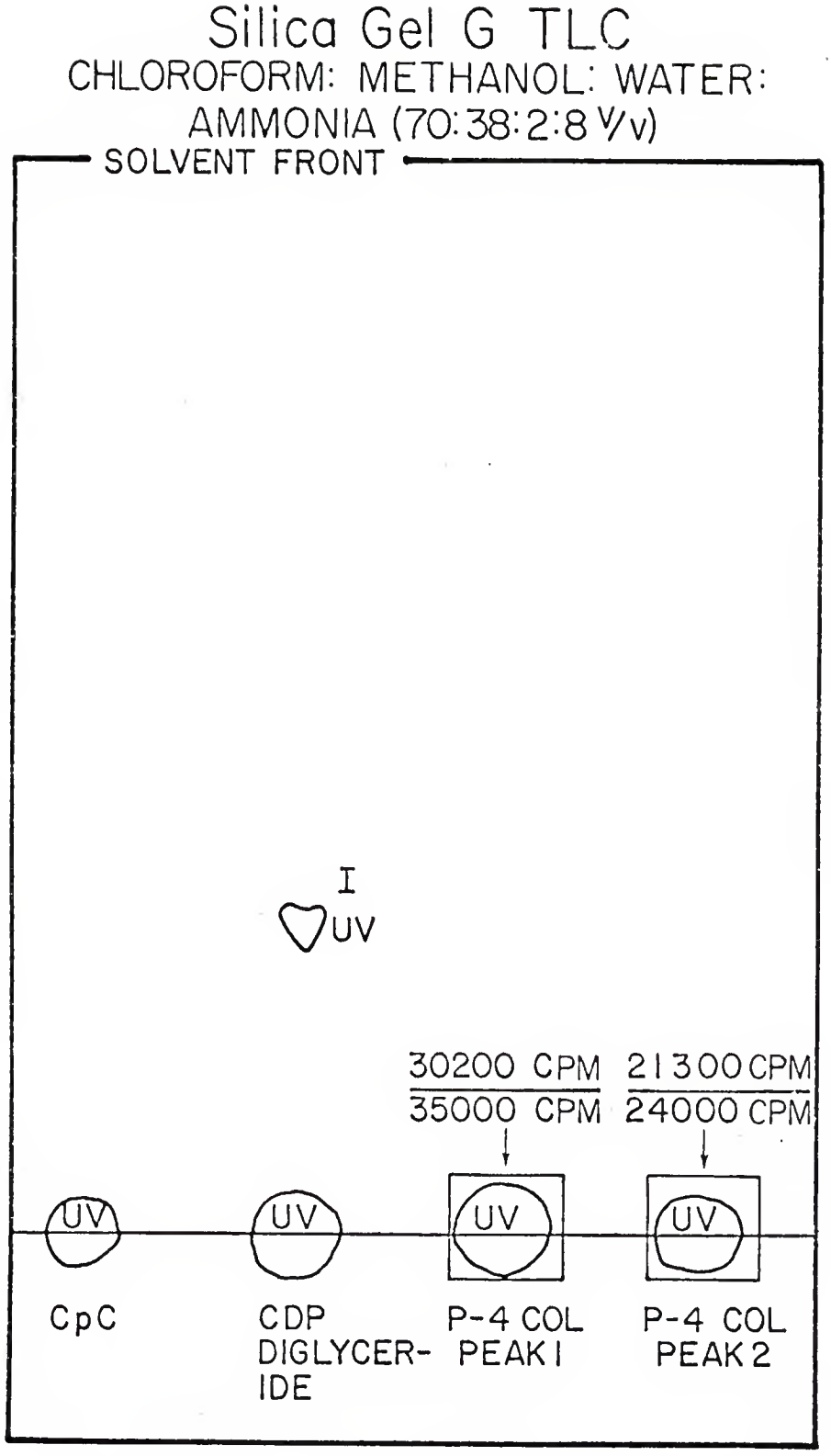
PHOSPHOLIPID EXTRACTION OF THE ^{3}H-CMP LABELED PRODUCT

Sample	Total CPM	% of Control
^{3}H-CTP product	58.50×10^{4}	-
First extraction of product in chloroform solution		
Chloroform phase	0.09×10^{4}	0.2
Aqueous phase	37.20×10^{4}	64.0
Second extraction of chloroform phase		
Chloroform phase	0.13×10^{4}	0.2
Aqueous phase	0.09×10^{4}	0.2
Third extraction of Chloroform phase		
Chloroform phase	0.19×10^{4}	0.3
Aqueous phase	0.27×10^{4}	0.4

A 0.1 ml sample of the ^{3}H-CMP-labeled product (containing 58.5×10^{4} cpm) obtained from nucleic acid extraction was dissolved in water and mixed with 1.0 ml of methonal containing 0.1N HCl. Ten minutes later 2.0 ml of chloroform was added to the mixture followed by 4 ml of 2M KCl. This was vigorously mixed on a vortex mixer for 5 minutes at 25°. The phases were allowed to separate upon standing at room temperature and then the aqueous phase was drawn off and the chloroform phase was extracted 2 more times. 100 µl samples of each phase were counted for radioactivity as described in the methods.

Figure 7. THIN LAYER CHROMATOGRAPHY OF THE ^{3}H-CMP LABELED PRODUCTS ELUTED FROM THE BIOGEL P-4 COLUMN

Ascending chromatography of the ^{3}H-CMP labeled products eluted from the Biogel P-4 column was performed according to the method of TerSchegett et al. (181) on silica gel G in closed tanks at room temperature. The solvent was chloroform; methanol; water; ammonia (70:30:2:8, v/v). The standards were $C_{p}C$ and CDP-diglyceride. Twenty microliter samples of the ^{3}H-CMP labeled products and standards were spotted 3.0 cm from the lower edge of the plate and dried before developing in the solvent. The 800 MW ^{3}H-CMP labeled product (BGP-1) spot contained 35,000 cpm and the 680 MW product (BGP-2) spot contained 35,000 cpm. This was determined by spotting the same volume of each product on a plate and then removing a 1.0 cm^2 section containing the spot and measuring its radioactivity in toluene scintillation fluid. The CDP-diglyceride was detected by ultraviolet light and by staining with iodine vapor. Radioactivity was detected by sequentially removing 1 cm^2 sections from the silica gel plate and counting them in toluene scintillation fluid.


Silica Gel G TLC
CHLOROFORM: METHANOL: WATER:
AMMONIA (70:38:2:8 V/v)
SOLVENT FRONT
I
UV
30200 CPM
35000 CPM
21300 CPM
24000 CPM
UV
UV
UV
UV
CpC
CDP DIGLYCER-IDE
P-4 COL PEAK 1
P-4 COL PEAK 2

which CDP-digylceride migrates (181). Ascending chromatography of the ^{3}H-CMP labeled products was carried out as described in the legend. The labeled products behaved similarly to C_pC in that they both failed to migrate in the system and they failed to stain with iodine vapor indicating that they contained no lipid moieties. As noted in Figure 7 100% of the cmp were recovered in the 1 cm square section comprising the origin. No significant radioactivity was detected in the other 1 cm squares which were sequentially removed from the plate and counted for radioactivity. The CDP-diglyceride standard migrated in the system and stained with iodine indicating that the radioactive products are not CDP-diglyceride.

Attempts were then made to ascertain the structure of the labeled products. This involved treating materials eluted from the P-4 column peaks with nucleases and analyzing the cleavage products by their migration in a PEI cellulose TLC system in which ribonucleoside monophosphates can be distinguished. Treatment of the labeled material (BGP-1 and BGP-2) eluted from the P-4 column peaks with snake venom phosphodiesterase resulted in 93% of the radioactive label migrating to a position corresponding to 5'-CMP (Figure 8 and 9), the expected cleavage products of poly C and C_pC. However, when the products were treated with RNase A, the radioactive label remained at the origin as did the untreated products. This data indicates that the products were insensitive to RNase A. This being the case, then the products have a chemical structure which is different from that of a homopolymer of CMP since RNase A cleaves C_pC to the expected 2', 3' CMP products. However, the possibility exists that these were homopolymers which have modified 3' terminus which prevents the RNase A from binding. The possibility of a small oligonucleotide with a 5'

Figure 8. THIN LAYER CHROMATOGRAPHY OF THE PRODUCTS OF THE ENZYMATIC CLEAVAGE OF THE ^{3}H-CMP LABELED MATERIAL ELUTED FROM THE P-4 COLUMN

Ascending chromatography of the products of the enzymatic cleavage of the ^{3}H-CMP labeled material eluted from the P-4 column was performed on PEI cellulose as described in the methods. The molecules eluted from the Biogel P-4 column BGP-1 (800 MW) and BGP-2 (680 MW) were treated separately with each of the digestive enzymes; snake venom phosphodiesterase, and ribonuclease A. The standards were C_pC, 2'-3'-CMP, and 5'CMP. Twenty microliters of each digest was spotted 3.0 cm from the bottom of the plate and dried. The enzymatic digest spots contained the cpm indicated in the denominators. Ribonucleoside monophosphates were chromatographed as described in the methods. The nucleotides were detected with ultraviolet light. Radioactivity was detected by sequentially removing 1.5 cm^2 sections of the PEI cellulose and counting them in toluene scintillation fluid.

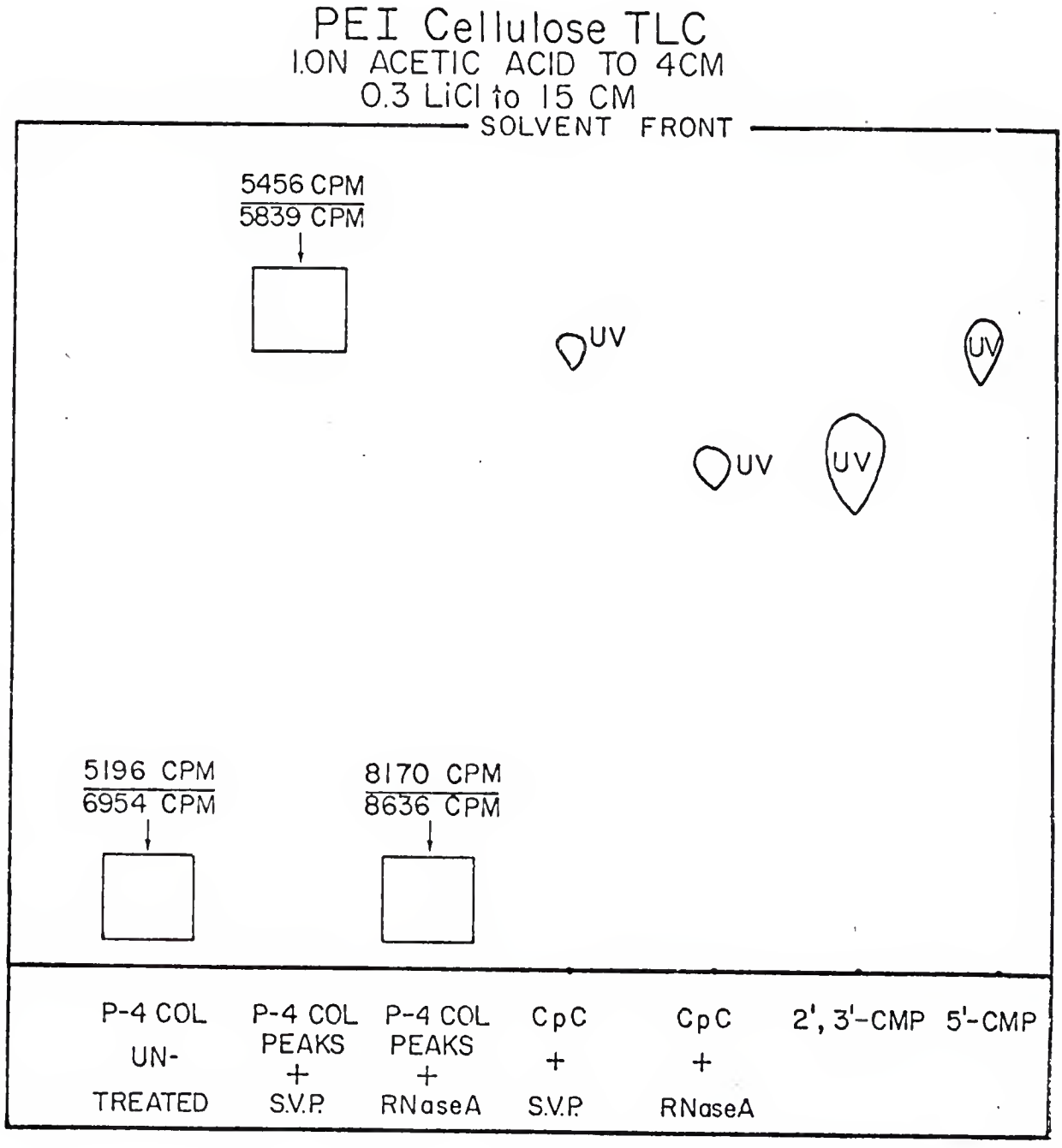
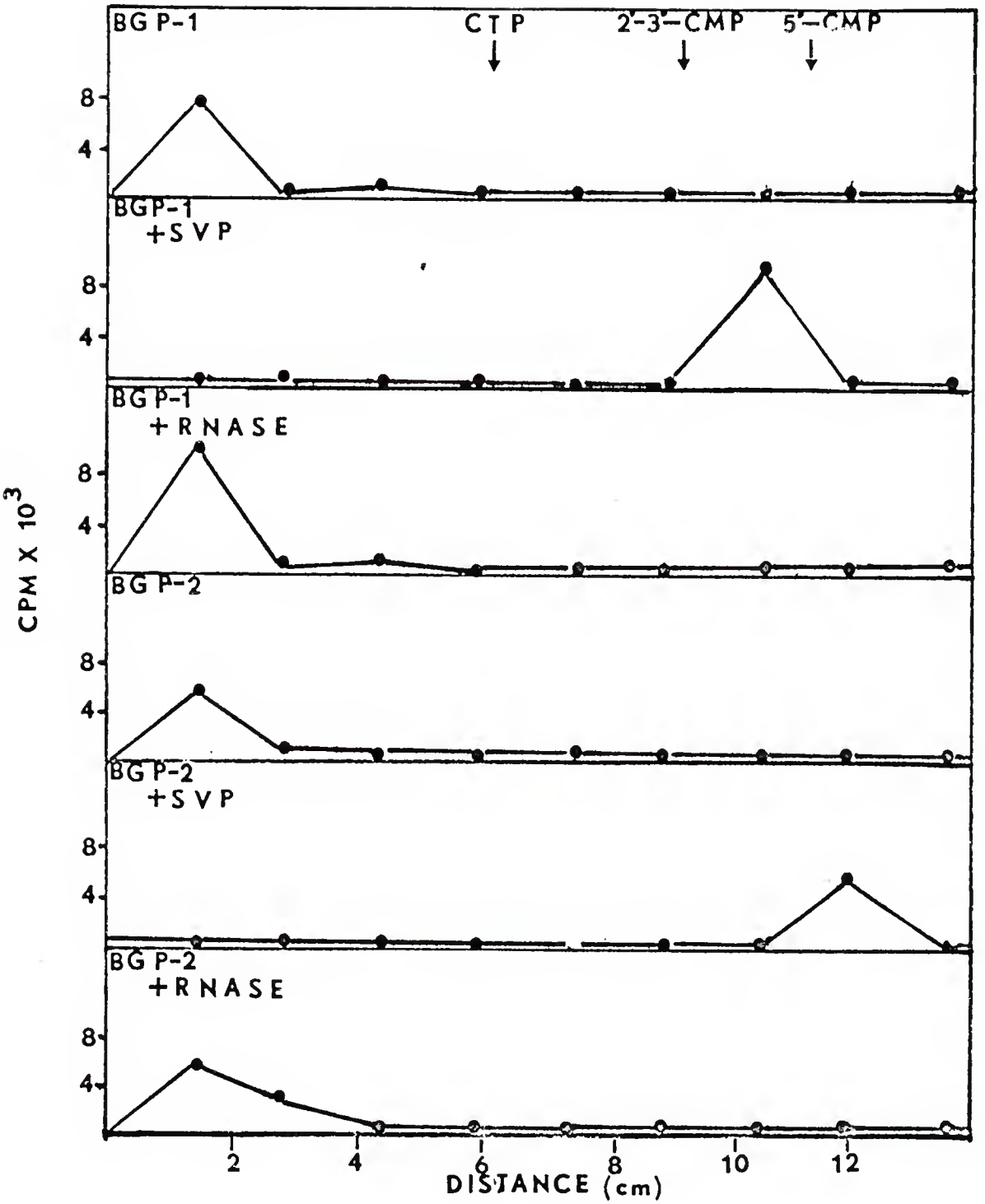

PEI Cellulose TLC
1.ON ACETIC ACID TO 4CM
0.3 LiCl to 15 CM
SOLVENT FRONT
5456 CPM
5839 CPM
UV
UV
UV
UV
5196 CPM
6954 CPM
8170 CPM
8636 CPM
P-4 COL UN-TREATED
P-4 COL PEAKS + S.V.P.
P-4 COL PEAKS + RNaseA
CpC + S.V.P.
CpC + RNaseA
2', 3'-CMP
5'-CMP

Figure 9. THE MIGRATION OF THE ENZYMATIC CLEAVAGE PRODUCTS CONTAINING TRITIUM LABEL ON PEI CELLULOSE

The migration of the enzymatic cleavage products containing tritium label on PEI cellulose as described in Figure 8.


BG P-1
CTP
2'-3'-CMP
5'-CMP
BGP-1
+SVP
BG P-1
+RNASE
BG P-2
BGP-2
+SVP
BG P-2
+RNASE
8
4
CPM X 10^3
2
4
6
8
10
12
DISTANCE (cm)

blocked terminus and/or methylated nucleoside moieties can not be ruled out.

The Solubilization and Partial Purification of Euglena Mitochondrial RNA Polymerase

The final objectives were to solubilize and purify the mitochondrial DNA-directed RNA polymerase, to characterize its activity, to determine its requirements for product synthesis, and to compare it to nuclear RNA polymerase activities with respect to these requirements. In order to accomplish this it was necessary to prepare mitochondria which were devoid of contaminating nuclear DNA. This was achieved by obtaining a crude 5P mitochondrial pellet, as described in Figure 1, and washing it twice in STM Buffer before incubating the mitochondria in the presence of deoxyribonuclease I (DNase I) for thirty minutes, zero degrees centigrade. These mitochondria were shown to be devoid of nuclear DNA by work substantiated in this laboratory and no further purification was necessary. The buoyant densities of the DNA in the mitochondrial preparations in CsCl are demonstrated in Figure 10. The crude washed 5P mitochondria yield mostly Euglena nuclear DNA (Tube a) which has a density of 1.707 g/cm^3 but after five minutes of incubation in DNase I at 4 ^{o}C, most of the nuclear DNA was removed (Tube b). Thirty minutes of incubation in DNase removes all of the contaminating nuclear DNA and only mitochondrial DNA remains, which has a density of 1.691 g/cm^3 (Tube c). Further treatment with DNase for one hour shows that the mitochondrial DNA is still present and is therefore protected from digestion by DNase I by the mitochondrial membranes. The mitochondria purified in this manner demonstrated the same ribonucleotide incorporating activities as the mitochondria purified on renografin gradients. Table 9 shows that the DNased treated mitochondria incorpora-

Figure 10. DENSITY EQUILIBRIA ANALYSIS OF DNA FROM DNASED MITOCHONDRIA

The density equilibria analysis of DNA in mitochondria purified by DNase treatment was measured by CsCl density gradient centrifugation as described in the methods section. The DNA standards were Cytophaga P-11 DNA with a density of 1.693 g/cm^3, Pseudomonas aeruginosa DNA with a density of 1.727 g/cm^3, and bacteriophage Φ25 DNA with a density of 1.742 g/cm^3. Euglena gracilis mitochondrial DNA has a density of 1.691 g/cm^3 and Euglena nuclear DNA has a density of 1.707 g/cm^3 in CsCl. The sources of the DNA were washed 5P mitochondria with DNA standards (tube a), the same mitochondrial preparation after treatment with DNase I for 5 min (tube b), after 30 min. of treatment in (tube c), after 1 hr. of treatment (tube d) and the DNA standards (tube e).

CsCl Density Gradient Gels
DNA STAINED WITH ETHIDIUM BROMIDE

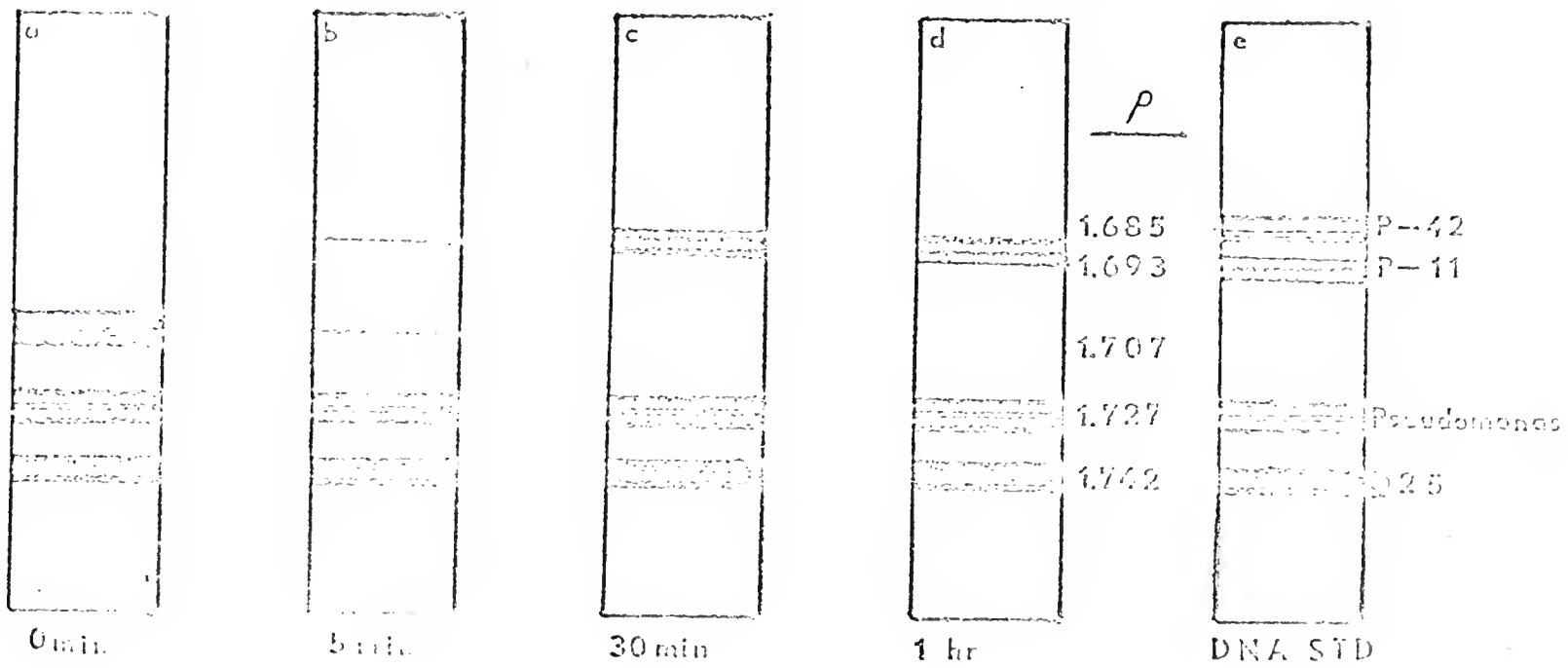


TABLE 9

A COMPARISON OF THE INCORPORATION OF ^{3}H-UTP and ^{3}H-CTP BY ISOLATED MITOCHONDRIA PURIFIED BY TREATMENT WITH DNASE I

Labeled Ribonucleotide	Reaction Mixture	CPM
^{3}H-UTP	Complete	1,624
^{3}H-UTP	-DNA	869
^{3}H-CTP	Complete	27,364
^{3}H-CTP	-DNA	16,669

Mitochondria purified by DNase I treatment were treated with isotonic buffer to assure swelling and permability. The RNA polymerase assay was performed as described in the methods. Reaction mixtures of 0.2 ml contained 1.0 mM of each unlabeled NTP and 0.016 mM ^{3}H-UTP or ^{3}H-CTP, 2 Ci/mmole.

ted tridum from ^{3}H-CTP at a ten fold greater rate than ^{3}H-UTP. However, about fifty percent of the incorporating activities from both ^{3}H-CTP and ^{3}H-UTP are DNA dependent which is in contrast to the ^{3}H-CTP incorporating activities in renografin purified mitochondria which was not DNA dependent.

Solubilization of the Mitochondrial RNA Polymerase Activity

The difficulty of defining proper conditions for solubilization of the chloroplast RNA polymerase of Euglena gracilis (166,185) plus the rapid inactivation of mitochondrial RNA polymerase activities by some solubilization methods (11), prompted the investigation to determine optimum conditions for solubilizing the activity from Euglena mitochondria (Table 10). An approach used by Rogall and Wintersherger (15) were used to determine the optimum conditions for solubilization of the mitochondrial RNA polymerase. A volume of mitochondrial suspension in lysis buffer was added to an equal volume of the detergent-KCl solution to bring the mixture to the indicated final concentrations. The mixtures were centrifuged at 30,000 rpm to sediment non-solubilized material and the supernatants were assayed for RNA polymerase activities. The efficiencies of the different treatments in the solubilizations of RNA polymerase activity from the mitochondria are summarized in Table 10. It can be seen that the combined treatment with KCl and Triton X-100 produced the highest yield of activity solubilized. However, NP-40, a detergent employed by other investigators (15,173), and 0.5 M KCl was almost as effective. Treatment with 0.5 M KCl alone failed to solubilize significant RNA polymerase activity. Treatment with either detergent alone was significantly less than with the Triton X-100 plus 0.5 M KCl treatment which completely destroyed the succinic dehydrogenase

TABLE 10

RNA POLYMERASE ACTIVITY SOLUBILIZED FROM MITOCHONDRIA

Treatment of Mitochondria in Lysis Buffer	RNA Polymerase Activity (units/g wet wt.)	
	30KS	30KP
+ Water	4.2	69
+ 0.5 M KCl	4.2	63
+ 0.8% NP-40	45.5	46
+ 0.8% Triton X-100	34.0	37
+ 0.8% NP-40 + 0.5 M KCl	56.0	0
+ 0.8% Triton + 0.5 M KCl	68.0	15
+ Water (Sonication)	0	28

Mitochondria purified by DNase treatment were suspended in lysis buffer (1g wet wt/10 ml). Mitochondrial samples of 1.0 ml each were then added to an equal volume of the indicated lysis mixture which contained 0.01 M EDTA plus additions shown. The mixtures were mixed gently for 15 min. at $4^{o}C$ and then centrifuged for 90 min. at 30,000 rpm (Ti 50 rotor). The supernatants and pellets were dialyzed against TGMED Buffer for 4 hrs. before analyzing them for RNA polymerase activity as described in the methods. The concentration of ^{3}H-UTP was 0.016 mM with a specific radioactivity of 2 Ci/mmole.

activity (Table 11) in the supernatant. The NP-40 plus 0.5 M KCl treatment produced supernatants that contained succinic dehydrogenase activities as high as they were in the control mitochondrial pellet. This suggested that Triton X-100 detergent was effective in completely disrupting the inner membrane thus freeing the succinic dehydrogenase of the membrane associated lipids which are needed to reconstitute the activity. The NP-40 detergent does not seem to be able to free the succinic dehydrogenase enzyme from the associated lipid since any loss of activity in the pellet could be observed as activity in the supernate indicating membrane components are present in this fraction needed for the expression of succinic dehydrogenase activity. The NP-40 detergent even with 0.5 M KCl does not seem to be able to free the succinic dehydrogenase enzyme from the associated membrane components. It appears that although there is 80% as much RNA polymerase activity in the 30,000 rpm supernatant produced by NP-40 as there is in the triton treated supernatant, the NP-40 supernatant may not contain solubilized RNA polymerase but just small mitochondrial fragments that fail to sediment at 30,000 rpm in 90 minutes. Therefore, the 0.8% Triton X-100 treatment with 0.5 M KCl was used for solubilizing the mitochondrial RNA polymerase.

The Effect of Ammonium Sulfate Precipitation on Mitochondrial RNA Polymerase Activity

It has been reported that *Neurospora* mitochondrial RNA polymerase (11) activity could not survive ammonium sulfate precipitation, chromatography on DEAE-Cellulose, extensive dialysis, concentration by ultrafiltration, and sucrose gradient centrifugation. Therefore, it was necessary to determine if *Euglena* mitochondrial RNA polymerase could survive ammonium sulfate treatment because the selection of proper salt

TABLE 11

SUCCINIC DEHYDROGENASE ACTIVITY IN THE MITOCHONDRIAL SUPERNATANTS AND PELLETS AFTER SOLUBILIZATION TREATMENT

Treatment of Mitochondria in Lysis Buffer	Succinic Dehydrogenase Activity (Units x 10^{-3})	
	30KS	30KP
+ Water	0	40
+ 0.5 M KCl	0	49
+ 0.8% NP-40	22	27
+ 0.8% Triton X-100	0	31
+ 0.8% NP-40 + 0.5 M KCl	34	0
+ 0.8% Triton + 0.5 M KCl	0	0
+ Water (sonication)	0	0

A unit is a µmole of dichlorophenolindophenol reduced per minute per ml of enzyme sample at 25°C.

concentration would allow the fractionation of RNA polymerase away from other mitochondrial proteins, thus effecting another purification step.

The effect of ammonium sulfate on the mitochondrial RNA synthesizing activity was measured (Table 12) by adding to solubilized mitochondrial RNA polymerase samples ammonium sulfate to the desired concentration with mixing followed by centrifugation at 40,000 rpm for 90 minutes. The RNA polymerase activity was measured in the redissolved and dialysed protein samples as described in the methods. The results indicated that 90% of the activity was recovered when the ammonium sulfate concentration was 50%. It was interesting to observe that when the 30,000 rpm super (control) was centrifuged at 40,000 rpm for 70 min. 19% of the activity was lost. This could represent the activity that is not completely solubilized and thus sediments at 40,000 rpm but not at 30,000 rpm.

Solubilization and Purification Of Mitochondrial RNA Polymerase

The scheme for the purification of the mitochondrial RNA polymerase is shown in Figure 11. The optimum conditions described in Table 10 were used to solubilize mitochondrial RNA polymerase. The washed mitochondria obtained from 182g (wet weight) of cells were purified by treatment with pancreatic deoxyribonuclease (100 µg/ml) for thirty minutes. The mitochondria obtained after two washings in STE buffer were suspended in lysis buffer and either used directly for enzyme solubilization or stored in liquid nitrogen prior to use. An equal volume of lysis mixture was mixed with mitochondrial suspension to bring the final concentration to 0.8% Triton X-100, 0.50 M KCl, and 0.005M EDTA. The mixture was gently stirred for 15 minutes, 4^{o}C. The supernatant obtained after centrifuging at 30,000 rpm for 90 minutes was brought to 50% saturation with

TABLE 12

THE EFFECT OF AMMONIUM SULFATE PRECIPITATION ON MITOCHONDRIAL RNA POLYMERASE ACTIVITY

Treatment % $(NH_4)_2SO_4$ Saturation	Activity (CPM/ml)	% Recovered in Pellet
30K Super	12420	-
+ 0, 40KP	1915	19
+ 30, 40KP	5660	46
+ 40, 40KP	6640	53
+ 50, 40KP	11220	90
+ 60, 40KP	6220	50
+ 65, 40KP	8830	71
+ 70, 40KP	9880	80
+ 75, 40KP	9090	73
+ 80, 40KP	7520	61

Mitochondria purified by DNase treatment were suspended in lysis buffer (1 g wet wt/10 ml) and the RNA polymerase activity was solubilized as described in the methods. Five milliliters of the mitochondrial suspension were added to an equal volume of the lysis mixture (1.6% Triton X-100, 1.0 M KCl, and 0.01 M EDTA pH 7.9) and mixed gently for 15 min. at 4°C and then centrifuged for 90 min. at 30,000 rpm (Ti 50 rotor). The supernatants were divided into 1.0 ml aliquots and solid ammonium sulfate was added to each aliquot to the desired concentration. The ammonium sulfate was dissolved at 4°C by gently mixing and the precipitated protein was collected by centrifugation at 40,000 rpm, 70 min. (Ti 50 rotor). Each pellet was dissolved in 1.0 ml of TGMED buffer and dialyzed against 2 liters of TGMED for 4 hrs. at 4°C. The ammonium sulfate concentration in each dialysate was determined with the conductance bridge and the salt concentration was adjusted to 0.05 M ammonium sulfate. The RNA polymerase assay was performed as described in the methods except that KCl was omitted.

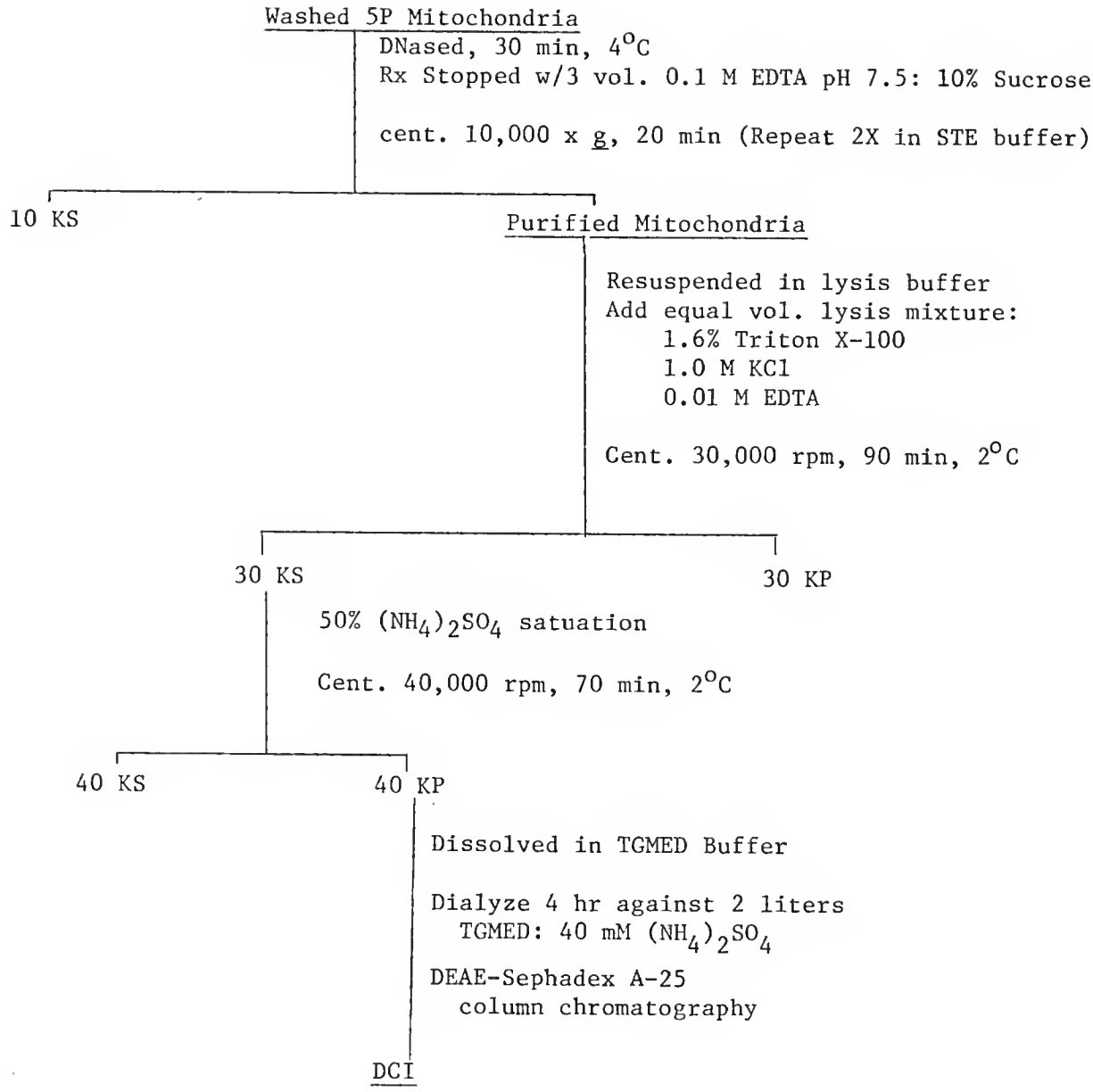


Figure 11. PROCEDURE FOR THE PARTIAL PURIFICATION OF MITOCHONDRIA RNA POLYMERASE

respect to ammonium sulfate. The precipitation collected at 40,000 rpm was dissolved in TGMED buffer and dialysed against TGMED containing 50 mM ammonium sulfate. The dialysate was subjected to DEAE-Sephadex chromatography and the eluted fractions were assayed for ^{3}H-CTP incorporating activities using the RNA polymerase assay (Figure 12). The void volume contained an activity which incorporated tritium preferrentially from the substrate ^{3}H-CTP by a reaction which was not dependent upon added DNA or the other Ribonucleotide substrates into acid insoluble products which resisted pancreatic ribonuclease digestion (Table 13). However, an activity which incorporated label from ^{3}H-UTP into acid insoluble and RNase digestible products was eluted from the DEAE-Sephadex column (Figure 12) in a single peak between 0.32M and 0.37M ammonium sulfate in TGMED buffer. The specific activity of the RNA polymerase after this purification step was 0.3 nmoles UMP incorporated/mg protein (estimated from A_{280} absorbancy)/10 minutes at 37^{o}C. This enzyme activity demonstrates a dependence upon added DNA and ribonucleoside triphosphate substrates (Table 14) for the incorporation of label from ^{3}H-UTP. The mitochondrial RNA polymerase activity is also inhibited by low concentrations (10 µg/ml) of actinomycin D (Table 15). The activity is insensitive to rifampin, α-amanitin, streptovaricin, rifamycin AF/013 and rifamycin AF/05. Attempts to concentrate this activity by rechromatography on a smaller DEAE-Sephadex A-25 column or to further purify the activity by glycerol gradient centrifugation resulted in the loss of significant activity.

Figure 12. ION EXCHANGE CHROMATOGRAPHY ON DEAE-SEPHADEX A-25

The procedure is described in the methods. The RNA polymerase activity profile when ^{3}H-UTP was substrate is represented by the solid line while the profile when ^{3}H-CTP was the substrate is represented by the broken line (——Δ——Δ——). The A_{280} profile is represented by the double line while the ammonium sulfate concentration in each fraction is represented by the heavy dashed line.

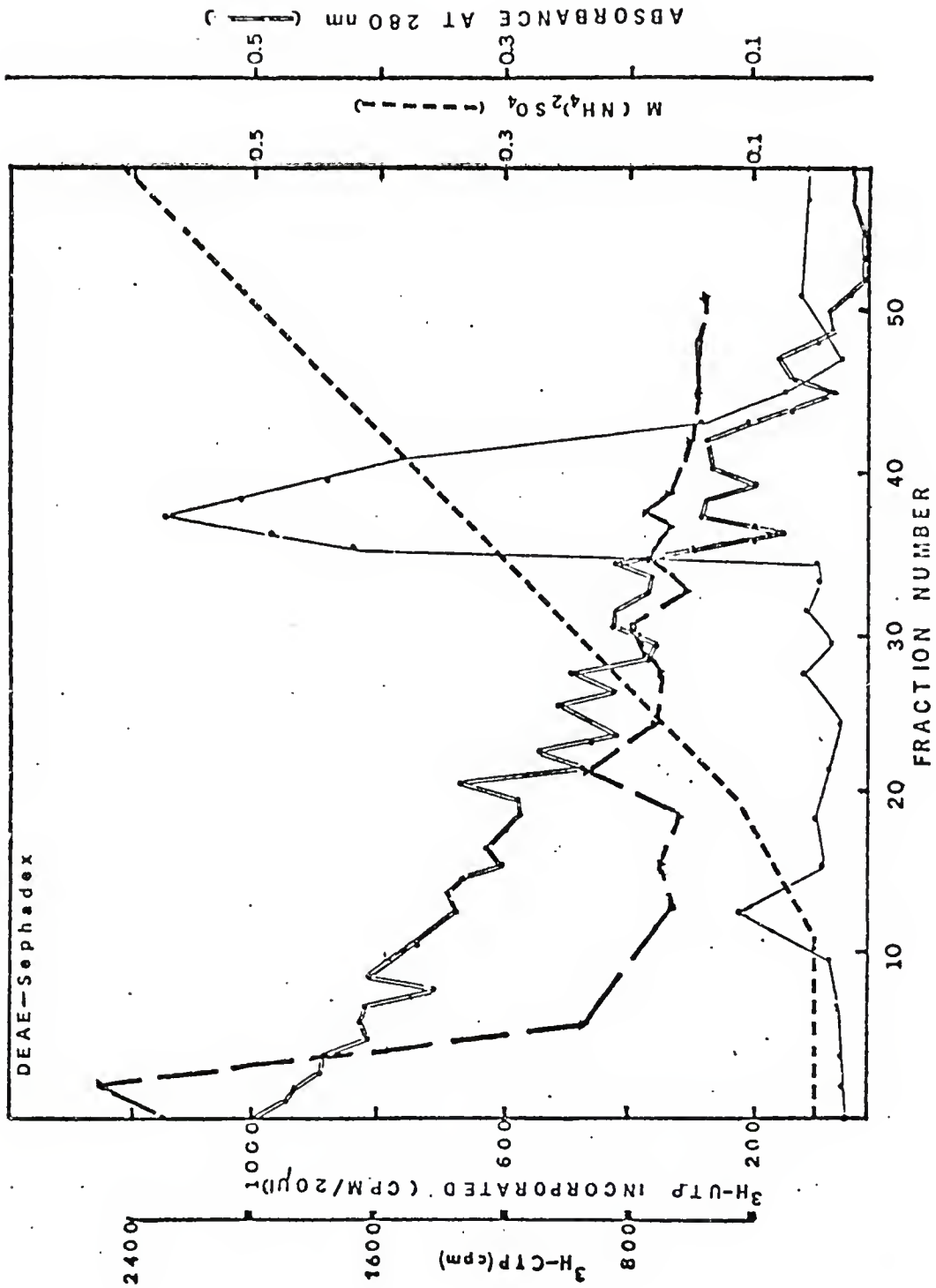

DEAE—Sephadex
FRACTION NUMBER
10
20
30
40
50
^{3}H-UTP INCORPORATED (CPM/20µl)
200
600
1000
^{3}H-CTP(cpm)
800
1600
2400
M $(NH_4)_2SO_4$ (– – – –)
ABSORBANCE AT 280 nm (——)
0.1
0.3
0.5

TABLE 13

A COMPARISON OF TRITIUM INCORPORATION FROM ^{3}H-UTP and ^{3}H-CTP BY THE ACTIVITY IN THE DEAE-SEPHADEX A-25 COLUMN FRACTION 3

Reaction Mixture	CPM
^{3}H-UTP	
Complete	200
^{3}H-CTP	
Complete	5428
-DNA	2581
-NTP	4389
+RNase	5316

The RNA polymerase assay reaction mixtures contained 95 µl of the void volume activity in a total volume of 0.20 ml. The concentration of ^{3}H-CTP, were 0.016 mM, 2 Ci/mmole.

TABLE 14

THE DEPENDENCY OF THE DEAE COLUMN FRACTION 38 INCORPORATION OF ^{3}H-UMP UPON ADDED RIBONUCLEOSIDE TRIPHOSPHATES AND DNA

Additions or Deletions	CMP	Percent of Control
Complete	1488	-
-DNA	187	13
-NTP	398	27
-ATP	168	11
-CTP	238	16
-GTP	178	12
+ Pancreatic RNase (100 µg/ml)	355	24

The RNA polymerase assay reaction mixtures contained 20 µl of DCI in a total volume of 0.20 ml. The concentration of ^{3}H-UTP was 0.016 mM, 2 Ci/mmole.

TABLE 15

INHIBITORS OF ^{3}H-UTP INCORPORATION BY THE DEAE COLUMN FRACTION 38

Inhibitors	CPM	% Activity
Distilled Water	2496	100
1% DMF	2521	100
Actinomycin D, 10 µg/ml	82	3
Rifampin, 100 µg/ml	1898	76
Rifamycin AF/013, 100 µg/ml	2109	85
Rifamycin AF/05, 100 µg/ml	2065	83
Streptovaricin, 100 µg/ml	2369	95
α-Amanitin, 5 µg/ml	1490	60
Pancreatic RNase 100 µg/ml	69	3

The RNA polymerase assay was performed with the addition of inhibitors to the final concentrations indicated above. The concentration of ^{3}H-UTP was 0.016 mM, 2 Ci/mmole. Rifampin, rifamycin AF/013, rafamycin AF/05, and streptovaricin were solubilized in 1% N'N' dimethyl formamide. Reaction mixtures contained 50 ml of the DCI enzyme in a total volume of 0.2 ml.

Enzymatic Properties of the
Mitochondrial RNA Polymerase

The dependence of the mitochondrial RNA polymerase on the concentration of $MnCl_2$ is shown in Figure 13. Low concentration (about 1 mM) of Mn^{++} stimulated the activity of this enzyme and higher concentrations decreased the activity. However, the enzyme activities were also stimulated by low concentrations of Mg^{++} (Figure 14) demonstrating the optimal concentration at 0.5 mM.

The template requirement of the mitochondrial enzyme is summarized in Table 16. The mitochondrial enzyme prefers poly d(AT) as a template which is similar to the rifampicin sensitive mitochondrial RNA polymerase from Neurospora crassa, described by Küntzel and Schäfer (11). It is interesting to note that while Euglena nuclear DNA was a more effective template than denatured calf thymus DNA that it is not necessarily a more preferred template than Euglena mitochondrial DNA. The mitochondrial DNA was only available in 1/5 the saturating DNA concentration and a comparison of the templates was not possible.

Figure 13. DEPENDENCE OF THE ACTIVITY OF THE DEAE FRACTION 38 ON THE CONCENTRATION OF Mn^{++}

Assays were carried out as described in methods. The concentration of ^{3}H-UTP was 0.016 mM, 2 Ci/mmole. The reaction mixtures contained 20 µl of DCI enzyme in a total volume of 0.2 ml.

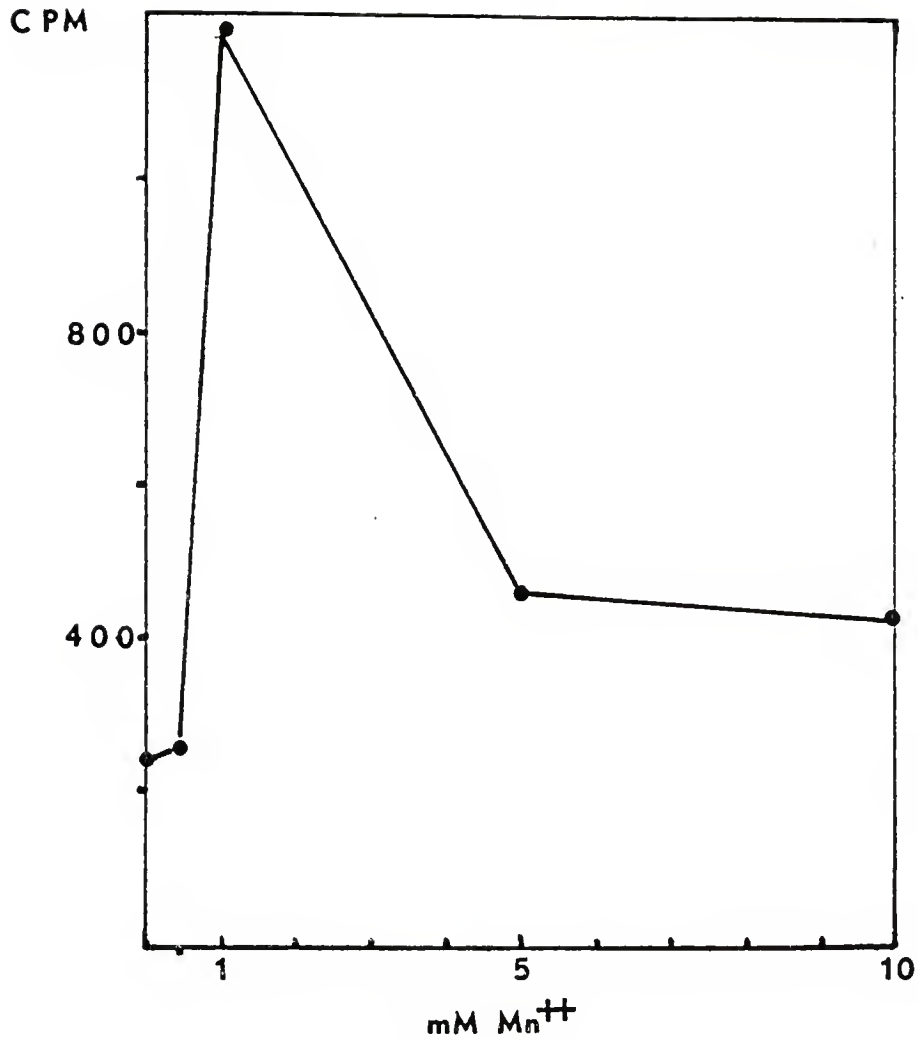

CPM
800
400
1
5
10
mM Mn++

Figure 14. DEPENDENCE OF THE ACTIVITY OF THE DEAE FRACTION 38 ON THE CONCENTRATION OF Mg^{++}

Assays were carried out as described in methods. The concentration of ^{3}H--UTP was 0.016 mM, 2 Ci/mmole. The reaction mixtures contained 20 µl of DCI enzyme in a total volume of 0.2 ml.

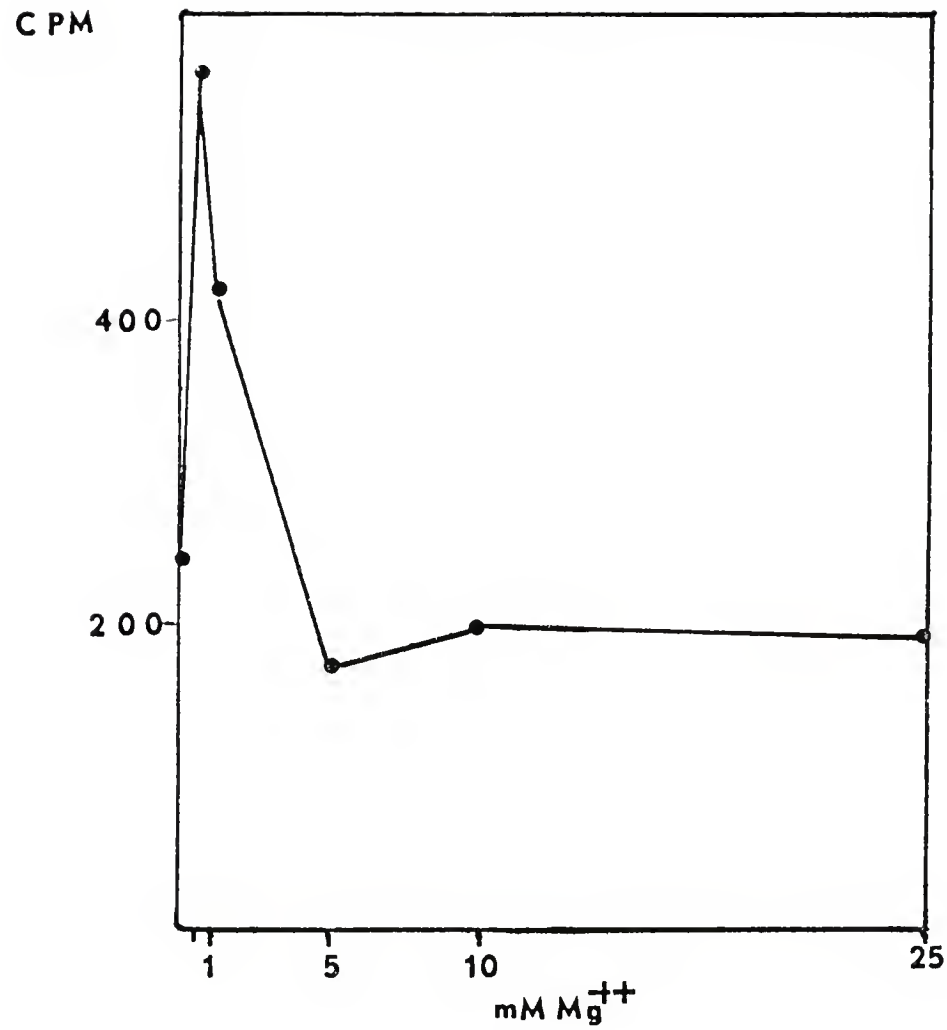

CPM
400
200
1
5
10
25
mM Mg++

TABLE 16

TEMPLATE SPECIFICITY OF THE DEAE COLUMN FRACTION 38

DNA Template	Units/ml	Percent of Control
Standard Assay Denatured calf thymus DNA	19	100
Poly d(AT)	45	236
Native Euglena nuclear DNA	23	121
Native E. coli DNA	14	73

Assays were carried out as described in materials and methods. Results are expressed as percent of the activity measured in the standard assay mixture with denatured calf thymus DNA as template. The DNA concentration was 50 µg per ml in each reaction. The ^{3}H-UTP concentration was 0.016 mM, 2 Ci/mmole.

DISCUSSION

This study was undertaken with the intentions of examining the mechanism by which Euglena gracilis, a eukaryotic cell, controls mitochondrial development. Since the DNA dependent RNA polymerase has been implicated in controlling development in prokaryotic systems it was felt that the mitochondrial RNA polymerase could be a major factor controlling development of the organelle. Therefore, the mitochondrial RNA synthesizing activities were studied with a view toward elucidating the components of the transcriptional process, in particular characterizing the mitochondrial DNA dependent RNA polymerase. The RNA synthesizing activity was first studied in isolated mitochondria because it was felt that it was necessary to characterize such general properties as sensitivity to inhibitors, types of RNA being synthesized, precursor requirements, metallic ion requirements, and temperature optimum. The streptomycin bleached aplastidic mutant was used for these studies because it provided a means by which processes controlling mitochondrial development could be distinguished from those controlling chloroplast development.

Mitochondria were isolated by a procedure that selected for the most enzymatically active organelles (33) based on succinic dehydrogenase activity. This procedure also provided purified mitochondria that contained mostly mitochondrial DNA (70%) based on the buoyant densities in CsCl (Figure 2). Although some contaminating nuclear DNA was in the preparation it represented less than 1% of the total nuclar DNA originally

present in the cell. This procedure provided the cleanest mitochondria feasible without treating them with an exogenous nuclease.

The incorporation of label from ^{3}H-ribonucleoside triphosphates into acid insoluble products showed no dependence upon the presence of the other ribonucleotides or that of added DNA. The cpm incorporated with ^{3}H-ATP or ^{3}H-GTP as substrates were so low that it made it difficult to determine the dependence of their incorporation upon the presence of the other ribonucleotides. The presence of mitochondrial DNA and mitochondrial pools of endogenous ribonucleotides could account for these results. However, ^{3}H-CTP was the preferred substrate compared to the other tritium labeled ribonucleoside triphosphates. The kinetics of the incorporation (Figure 3) allow the presence of nuclease activity in the mitochondria as demonstrated by departure from linearity after 5 to 10 min. With ^{3}H-UTP as substrate these mitochondria are capable of carrying out the synthesis of a product which demonstrates some sensitivity to RNase. The failure of the labeled product, obtained upon incubation with ^{3}H-CTP, to be degraded by pancreatic RNase A may be due to either the failure of the nuclease to get into the mitochondria or the alteration of the RNA 3' terminus thus preventing the RNase A from binding to the products. It is also possible that the product is complexed with DNA such that it is RNase insensitive. The results in Figure 3 also suggest that RNA synthesis based upon at least partial sensitivity of product to RNase could be better studied by following the incorporation of UTP by the isolated mitochondria rather than CTP.

The ^{3}H-CTP incorporating activity appears to be associated with mitochondrial DNA in that the activity is enriched 125 fold per μg of

DNA in the purified mitochondria (Table 2). If the activity is associated with DNA, then it is with mitochondrial DNA and not nuclear DNA, 95% to 99% of which has been removed by the purification procedure. This does not eliminate the possibility that the activity is associated with the mitochondrial membrane since the activity is enriched 8.7 fold per mg of protein in the purified mitochondria. This would be probable because the crude mitochondria may be contaminated more with nuclear DNA than with exogenous protein and enrichment observed may just reflect the extent to which the contaminating molecules have been removed.

The inhibition of the CMP incorporating activity by actinomycin D (Table 3) suggests that a DNA dependent reaction may be involved which would implicate either the RNA or the DNA polymerases. The use of ribonucleotide precursors eliminate DNA synthesis as a consideration since it is difficult to imagine the conversion of these precursors to deoxyribonucleotides in the period of the assay. The inhibition of the CMP incorporating activity by pyrophosphate indicates that the reaction involves a pyrophosphorolysis, implicating a RNA polymerase type reaction. However, phospholipid biosynthesis could not be ruled out because a pyrophosphorolysis reaction is involved in its utilization of CTP.

The acid insoluble CMP incorporating product is sensitive to snake venom phosphodiesterase as well as to alkaline hydrolysis. The specificity of the enzyme for attacking the phosphodiester bond to yield 5' nucleoside monophosphates and also the sensitivity of the phosphodiester bond to alkaline hydrolysis certainly indicates that the CTP product contained phosphodiester bonds. The properties demonstrated by the product were similar to those reported by Cherry et al. (184) for a poly C RNA polymerase activity in sugar beet nuclei. This activity was in-

hibited by actinomycin D and pyrophosphate while the product was insensitive to pancreatic RNase digestion yet it was sensitive to snake venom phosphodiesterase and alkaline hydrolysis. However, they were able to show by nearest neighbor analysis that the product was poly C.

The products of the CTP incorporating activity for this study were isolated by phenol extraction followed by ethanol precipitation of the aqueous phase. The size of the isolated products as determined by their elution position from a Biogel P-4 column indicated they comprised two classes of molecules with mean molecular weights of 800 and 680 respectively (Figure 4). These molecules have absorbancy spectra very similar if not identical to that of C_pC which suggests that cytosine is the only base incorporated into the products. The snake venom phosphodiesterase clevage product of both molecular weight classes is 5'-CMP, the expected clevage product of poly C of C_pC. The CTP incorporating products showed no sensitivity to pancreatic RNase A. The possibility exists that these molecules are di- or trinucleotides of CMP which have modified 3' terminus which prevents the RNase A from binding to the molecules. All attempts to demonstrate that the products of the ^{3}H-CTP incorporating activity could be CDP-diglyceride gave negative results.

Recent studies have demonstrated the presence of an unusual structure ($^7G^{5'}ppp^{5'}N^mpM^m_p$) at the 5' termini of a wide variety of eukaryotic and viral messenger RNA's in which the 5' end of the messenger RNA is blocked by a 7-methyl guanosin linked to a 2'0-methylated nucleotide, N^m, through a 5'-5' pyrophosphate bond (186,187). The 2'-0-methylated nucleotide N^m is linked to the adjacent nucleotide M^m by a 3'5' phosphodiester bond. These blocked, methyoated5'-termini ("caps") are resistant to digestion by ribonuclease. The possibility exists that the labeled products obtained

after incubating Euglena mitochondria with ^{3}H-CTP may have similar structures with cytidine as the methylated nucleoside. It is impossible to determine the assigned role of these small C-labeled molecules in the mitochondria without knowing their precise structures.

An activity which incorporated lable from ^{3}H-CTP was separated from the mitochondrial RNA polymerase when it eluted in the void volume during DEAE-Sephadex chromatography. This activity was similar to the activity observed in the isolated mitochondria in that it preferentially incorporated label from ^{3}H-CTP by a reaction which was not DNA dependent and did not require the other ribonucleotide substrates of RNA synthesis. The products of this activity were insensitive to pancreatic ribonuclease digestion. This data provides evidence that this activity is a mitochondrial associated activity; however, it may not be a RNA polymerase activity but this conclusion must be reserved until the identity of the product is determined.

The mitochondrial RNA polymerase of Euglena had not previously been solubilized and this study represents the first time that an RNA synthesizing activity has been solubilized and partially purified from a cytoplasmic organelle of this organism. The mitochondria used for solubilization of the mitochondrial enzyme were treated with DNase I to remove all contaminating nuclear DNA (185) and its associated proteins. The results demonstrated that it was possible to define optimum conditions for solubilizing the RNA synthesizing activities. The Triton X-100 detergent KCl treatment was effective in distrupting the mitochondrial membranes and releasing the RNA polymerase activity (Table 9) which could be fractionated by ammonium sulfate precipitation. The enzyme activity is also able to survive dialysis and DEAE-Sephadex chromatography, eluting in a single peak between 0.32

and 0.37 M $(NH_4)_2SO_4$ (Figure 11). This enzyme is different from the Euglena nuclear RNA polymerase II which elutes between 0.18 and 0.21 M $(NH_4)_2SO_4$ and is sensitive to α-amanitin. The mitochondrial enzyme is insensitive to α-amanitin and rifampicin and demonstrates a DNA dependence bases upon actinomycin D inhibition and the requirements for added DNA and all of the ribonucleotide substrates. Poly d(AT) is the preferred template and optimal activity is observed with low concentrations of Mn^{++} and Mg^{++}.

Attempts to concentrate the mitochondrial enzyme by rechromatography on a smaller DEAE-Sephadex column or to further purify the activity by gylcerol gradient centrifugation resulted in the loss of significant activity. This loss was probably due to the prolonged time at $4^{o}C$ necessary for these additional purification steps.

CONCLUSIONS

Euglena mitochondria contain a RNA polymerase activity which can be solubilized and then partially purified by DEAE-Sephadex chromatography. This enzyme is DNA dependent, requires the four ribonucleoside triphosphates and is not inhibited by rifampin. The mitochondrial enzyme is distinctly different from the nuclear RNA polymerase II (167,168) based on its elution from DEAE-Sephadex chromatography and sensitivity to inhibitors. The stability and yield of the mitochondrial enzyme is such that large preparations of mitochondria are required for further enzyme purification and characterization of the activity and molecular substructure (30 liters of log phase cells yield 53 units of mitochondrial activity per milliter of partially purified enzyme with a specific activity of 0.3 nmoles/mg protein).

Euglena mitochondria also contain an enzyme activity which preferentially incorporates CMP from CTP into small molecules of unknown structure, with molecular weights between 800 and 600 daltons. These molecules contain phosphodiester bonds but they are resistant to cleavage by pancreatic ribonuclease.

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BIOGRAPHICAL SKETCH

George Erwin Brown was born August 12, 1939 in Houston, Texas. He lived with his parents, Marshall Vernon Brown and Libbie Nickerson Brown; two sisters, Ida and Doris; and a brother, Marshall Jr., until he graduated from Prairie View High School, Prairie View, Texas. He received the Bachelor of Science Degree in Agricultural Education from Prairie View A&M College, Prairie View, Texas, May 1960. He did post graduate work in biology at Prairie View until he entered the U. S. Army as a commissioned officer in June 1961 where he served as a signal corps officer until July 1963. He taught High School Biology in the Hull-Daisetta School District and then the Royal Independent School District, both in Texas, until August 1966 when he assumed the position of a county agricultural agent for the Texas Agricultural Extension Service. He remained in this capacity until February 1969 when he became Director of an Adult Education Center for Harris County Department of Education. In September 1969, he entered the University of Florida on a Rockefeller Foundation Fellowship and in June 1970, he entered the Graduate School of the University of Florida, as a teaching assistant in the Department of Microbiology. This is when he met Dr. James F. Preston who has kept him busy every since. He received the Master of Science Degree in June 1972 and is now a candidate for the Doctor of Philosophy Degree in August 1976.

George is married to the former Carolyn M. Rogers and they have two children, a son, George Jr., age 5; and a daughter, Erika, age 2. George

is a member of the American Society for Microbiology, the National Institute of Science, and Beta Beta Beta Scientist Society. He is presently an Assistant Professor of Biology at Prairie View A&M University, Prairie View, Texas.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James F. Preston, III, Chairman
Associate Professor of Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the Department of Microbiology in the College of Arts and Sciences and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1976

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